

STUDIES ON COMPLEMENT AND ANTI-IMMUNOGLOBULINS

**Development of hemolysis in gel and
immunoenzyme techniques**

Lennart Truedsson



Lund 1984



STUDIES ON COMPLEMENT AND ANTI-IMMUNOGLOBULINS

Development of hemolysis in gel and
immunoenzyme techniques

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras å Institutionen för Medicinsk Mikrobiologi, Sölvegatan 23, Lund, torsdagen den 22 mars 1984 kl 9.15,

av
Lennart Truedsson
Leg.läk., Ssk

Organization LUND UNIVERSITY Department of Immunology Institute of Medical Microbiology	Document name DOCTORAL DISSERTATION	
	Date of issue 1984-03-22	
	CODEN: LUMEDW/(MEMI-1006)/1-57(1984)	
Author(s) Lennart Truedsson	Sponsoring organization	
Title and subtitle STUDIES ON COMPLEMENT AND ANTI-IMMUNOGLOBULINS Development of hemolysis in gel and immunoenzyme techniques		
Abstract A screening procedure, with two hemolysis in gel (HIG) assays, for detection of complement (C) deficiency states was evaluated. HIG with sensitized sheep erythrocytes (EA) was used for the classical pathway and HIG with guinea pig erythrocytes for the alternative pathway, both assays being capable of detecting defects in the terminal C sequence. The screening procedure was useful in adults, but not in newborns. Anti-immunoglobulins inhibited lysis in the classical pathway assay. Thus, rheumatoid factors (RF) in 9/37 rheumatoid arthritis (RA) sera, and antibodies to rabbit IgM in 14/21 IgA deficient sera gave patterns of inhibited lysis in the test with EA. Based on these observations, HIG assays for measurement of C activating RF were developed. RF that mediated lysis of EA prepared with reduced/alkylated IgG antibody were mainly of IgM class, as suggested by correlation with IgM-RF, but not IgG-RF or IgA-RF measured by ELISA. RF concentrations did not correlate with C1 activation in RA sera. Factor D of the alternative pathway was purified from plasma with 30 % yield. D was measured in serum and plasma by enzyme amplified electroimmunoassay. The 95 % geometric confidence interval for D in 144 adults was 75-143 in per cent of a normal serum pool, containing 1.6 mg/l. The values correlated with D measured with functional assay. By electroimmunoassay, 16 C components were measured in serum (C1q, C1r, C1s, C1 IA, C2, P, D, I, H, C6 and C7) or in EDTA-plasma (C4, C3, B, C5 and C8) in 100 newborns and their mothers. Newborns showed low concentrations of most components, the levels of B, P, C6 and C8 being lowest. C7 and D concentrations were higher than in adults. The majority of the C components showed raised concentrations in the maternal sera.		
Key words complement; complement screening; hemolysis in gel; complement components; cord levels; factor D; anti-immunoglobulins; electroimmunoassay; immunoenzyme techniques; rheumatoid factor; rheumatoid arthritis; IgA deficiency		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language English
ISSN and key title		ISBN 91-7222-690-0
Recipient's notes	Number of pages 134	Price
	Security classification	

Distribution by (name and address) Lennart Truedsson, Institute of Medical Microbiology
 Sölvegatan 23, S-223 62 Lund, Sweden

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature

Lennart Truedsson

Date 1984-02-10

STUDIES ON COMPLEMENT AND ANTI-IMMUNOGLOBULINS

**Development of hemolysis in gel and
immunoenzyme techniques**

**by
Lennart Truedsson**

**Department of Immunology, Institute of Medical Microbiology,
University of Lund
Lund, Sweden**

Lund 1984

© Lennart Truedsson
ISBN 91-7222-690-0
Printed in Sweden
Infotryck ab
Malmö 1984

To Ann, Emma, Malin and Erik



C O N T E N T S

List of original papers	6
Nomenclature and abbreviations	8
INTRODUCTION	9
Biochemistry of complement	10
Complement activation	12
Biological activities of complement	15
Biosynthesis and complement metabolism	16
Genetics and inherited abnormalities of complement components	16
Acquired complement aberrations and disease	17
Methods of measuring complement	18
Rheumatoid factors	19
Interactions between rheumatoid factors and complement	20
THE PRESENT INVESTIGATION	22
1. Screening for complement deficiencies by hemolysis in gel (HIG)	23
-Discussion	28
2. Complement activation by rheumatoid factors	31
-Discussion	33
3. Measurement of complement components	35
-Discussion	38

CONCLUDING REMARKS	41
SUMMARY	42
ACKNOWLEDGEMENTS	44
REFERENCES	45
APPENDIX: Original papers (I - VII)	59

Original papers

This thesis is based on the following papers referred to by Roman numerals in the text.

- I. Truedsson L, Sjöholm AG & Laurell A-B: Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. Acta path. microbiol. scand. Sect. C, 89:161-166, 1981.
- II. Truedsson L & Sjöholm AG: Inhibition of complement-mediated hemolysis in gel by rheumatoid factors. Acta path. microbiol. immunol. scand. Sect. C, 92:1-4, 1984.
- III. Truedsson L, Axelsson U & Laurell A-B: Frequent occurrence of anti-rabbit IgM in IgA deficiency. Acta path. microbiol. immunol. scand. Sect. C, 90:315-320, 1982.
- IV. Truedsson L, Sjöholm AG & Sturfelt G: Complement activating rheumatoid factors in rheumatoid arthritis studied by haemolysis in gel: relation to antibody class and response to treatment with podophyllotoxin derivatives. Submitted for publication.
- V. Truedsson L & Sturfelt G: Human factor D of the alternative pathway: purification and quantitation by enzyme amplified electroimmunoassay. J. Immunol. Methods, 63: 207-214, 1983.

- VI. Sturfelt G & Truedsson L: Complement factor D in normal sera: comparison of immunochemical and functional determinations. Acta path. microbiol. immunol. scand. Sect. C, 91:383-389, 1983.
- VII. Johnson U, Truedsson L & Gustavii B: Complement factors in 100 newborns and their mothers determined by electroimmunoassay. Acta path. microbiol. immunol. scand. Sect. C, 91:147-150, 1983.

Nomenclature and abbreviations

The nomenclature used for complement is that recommended by the World Health Organization (Austen et al., 1968; Alper et al., 1981). Enzymatic activity is designated with a bar over the component number - e.g., $\bar{C}1r$. Fragments arising from components on activation are denoted by the addition of a lower case letter to the symbol representing the component - e.g., C3a. Factors of the alternative pathway are designated by capital letters, e.g. D, B and so on.

ARA	American Rheumatism Association
CIC	circulating immune complexes
E	sheep erythrocytes
EAG	E sensitized with rabbit IgG antibodies
EAM	E sensitized with rabbit IgM antibodies
EAE	enzyme amplified electroimmunoassay
EDTA	ethylenediamine-tetra acetic acid
EGTA	ethyleneglycolbis(aminoethylether)-tetra acetic acid
ELISA	enzyme linked immunosorbent assay
GpE	guinea pig erythrocytes
GPS	guinea pig serum
HAE	hereditary angioedema
HIG	hemolysis in gel
MAC	membrane attack complex
NHS	normal human serum
RA	rheumatoid arthritis
RF	rheumatoid factors
VBS	veronal-buffered saline

I N T R O D U C T I O N

Complement is regarded as the principal humoral effector system of inflammation and host defense against microbial infection. Complement deficiency, whether inborn or acquired, is usually associated with disease, and suitable techniques and knowledge about complement in healthy individuals are essential for the assessment of complement in the clinical laboratory.

The bactericidal action of blood serum was first observed at the end of the 19th century (von Fodor, 1887; Nuttall, 1888). After the discovery of serum antibodies, it was shown that a serum substance (complement) was required for bacteriolysis to occur in the presence of specific antibodies (Pfeiffer & Issaëff, 1894). Bordet (1898) demonstrated lysis of sheep red blood cells by specific antibodies and complement. Evidence that complement was not a single substance was furnished by Ferrata (1907). Later, the classical complement system, as defined by immune hemolysis, was shown to comprise at least four components designated C'1, C'2, C'3 and C'4. Ueno (1938a, b) demonstrated that the reaction sequence was C'1, C'4, C'2 and C'3. That complement was in fact an enzyme system was suggested by Becker (1956), and by Lepow et al., (1956) who demonstrated the esterase activity of C'1.

An important contribution to complement research was made by Mayer, who developed the "one-hit hypothesis" for complement hemolysis (Mayer, 1961), according to which a single lesion of the cell membrane is sufficient for lysis.

When new chromatographic methods became available during the 1950's and 1960's, the components of the classical complement system were purified (cf. Müller-Eberhard, 1968; Rapp & Borsos, 1970). It became apparent that complement is an enzyme system of great biological significance (cf. Schur & Austen, 1968; Müller-Eberhard, 1969).

Pillemer and co-workers studied the mechanism of C3 depletion on incubation of serum with yeast and presented the concept of the properdin system (Pillemer et al., 1954). They found C3 depletion to be dependent on a newly discovered protein, pro-

perdin, and on two other serum factors, but independent of specific antibodies. However, the existence of the properdin system was not generally accepted until about 1970, when several investigators demonstrated an alternative activation mechanism of the complement system - i.e., activation of C3 - C9 without participation of C1, C2 or C4. (cf. Fearon & Austen, 1980; Müller-Eberhard & Schreiber 1980; Kazatchkine & Nydegger, 1982).

BIOCHEMISTRY OF COMPLEMENT (cf. Müller-Eberhard & Schreiber, 1980; Reid & Porter, 1981; Loos, 1982).

The complement components are all proteins, many of which are present in the body fluids as proenzymes. The plasma concentrations are very different for the various complement proteins (Table 1). Together they comprise about 15 % (w/w) of the normal plasma globulin fraction. The first component, C1, is a calciumdependent complex of three proteins, C1q, C1r and C1s (Lepow et al., 1963; Naff et al., 1964). The complex contains two subunits of C1r and C1s per molecule of C1q (Gigli et al., 1976). C1q has a hexameric structure with the globular region of each monomer connected by a collagen-like radial strand to a common stem region (Reid et al., 1972; Yonemasu & Stroud, 1972). C4 is structurally very similar to C3, and whereas C4 has three chains (Schreiber & Müller-Eberhard, 1974) C3 like C5 has two polypeptide chains (Nilsson et al., 1975). C2 and B₁ are structurally similar (Kerr, 1979), as are C6 and C7 (Podack et al., 1979). D differs from the other components in several ways. It is a β -globulin consisting of a single polypeptide chain with a molecular weight of 24,000 daltons (Götze & Müller-Eberhard, 1976). Factor D is present in enzymatically active form in plasma (Lesavre & Müller-Eberhard, 1978). Some physico-chemical properties of the complement components are given in Table 1.

Table 1. The Complement Proteins.

Component	Mean serum concentration(mg/l)	Molecular weight	Electrophoretic mobility	Activation products
C1q	66	410,000	γ_2	
C1r	48	170,000	β	C1 $\bar{r}$
C1s	34	85,000	α_2	C1 $\bar{s}$
C4	553	210,000	β_1	C4a, C4b
C2	25	115,000	β_2	C2a, C2b
C3	1201	195,000	β_1	C3a, C3b
D	2	25,000	β	
B	200	93,000	β_2	Ba, Bb
P	25	184,000	γ_2	
C5	67	205,000	β_1	C5a, C5b
C6	62	128,000	β_2	
C7	54	121,000	β_2	
C8	54	155,000	γ_1	
C9	59	75,000	α	
C1 $\bar{I}$ IA	190	100,000	α_2	
I	35	100,000	β_1	
H	475	150,000	β_1	
C4bp	250	540,000- 590,000	β_2	
Anaphylatoxin inactivator	Trace	310,000	α	
S-protein	500	84,000		

Data from Austen, 1979; Kazatchkine & Nydegger 1982; Lachmann & Peters, 1982.

COMPLEMENT ACTIVATION

Classical pathway (cf. Porter, 1977; Loos, 1982). Activation of the classical pathway is initiated when C1 binds to immune complexes. Binding of the C1q subunit requires one IgM, or at least two IgG adjacent binding sites (Müller-Eberhard & Calcott, 1966; Borsos, 1971). C1 is activated by the immunoglobulin IgG1, IgG2, IgG3 and IgM, either in complex with antigen or physically aggregated. IgG4 has low affinity for C1q and does not activate C1. Modification of tryptophan residues (Allan & Isliker, 1974), or reduction and alkylation of rabbit IgG antibodies (Johnson & Hoffmann, 1981) abolish their C1 activating properties. Certain other chemical modifications of IgG lead to a loss of its ability to activate but not alteration of its ability to bind C1 (Cooper, 1983).

The binding site for C1q is in the CH2 domain of IgG (Ellerson et al., 1972), and in the CH4 domain of IgM (Hurst et al., 1974). Activation of C1q leads to cleavage of C1r, and an active enzyme, C1r is formed (Ziccardi & Cooper, 1976). The action of C1r on the proenzyme C1s results in the formation of another enzyme, C1s.

In addition to the immune complexes, several other substances such as polyanions, polyanion-polycation complexes, C-reactive protein complexes and certain viruses are capable of activating C1 (cf. Cooper et al., 1976; Gewurz & Lint, 1977; Loos, 1982).

C4 and C2 are the only natural substrates for C1s. C4 is cleaved into two fragments C4a and C4b (Patrick et al., 1970; Budzko & Müller-Eberhard, 1970). Some C4b molecules become bound to cell membranes or immune complexes by their labile binding sites (Law et al., 1980a). C2 in a Mg^{2+} -dependent complex with C4b is cleaved by C1s into the fragments C2a and C2b (Gigli & Austen, 1969). The complex thus formed is the classical C3 convertase, $C4b,2a$ with its enzymatic activity in the C2 portion of the complex (Polley & Müller-Eberhard, 1968). The catalytic site in C2a is capable of cleaving C3 into C3a and C3b (Müller-Eberhard et al., 1967; Bokisch et al., 1969).

Activation of C1 is controlled by C1 IA, which forms stable

complexes with C1r and C1s , thereby abolishing their enzymatic activities (Gigli et al., 1968; Harpel & Cooper, 1975). C1 IA also inhibits proteases not related to the complement system, such as plasma kallikrein, plasmin and activated Hageman factor (Ratnoff et al., 1969; Forbes et al., 1970).

The amount of formed C4b,2a is regulated by the lability of the convertase and by the control proteins factor I and C4bp. The binding of C4bp to C4b prevents that of C2 to C4b; it accelerates dissociation of C2a from C4b,2a already formed, and increases the susceptibility of fluid phase C4b to cleavage by I (Cooper, 1975; Scharfstein et al., 1978; Fujita et al., 1978; Gigli et al., 1979). Factor I is a proteolytic enzyme which cleaves C4b into two fragments, C4c and C4d (Shiraishi & Stroud, 1975).

Alternative pathway (cf. Fearon & Austen, 1980; Müller-Eberhard & Schreiber, 1980; Kazatchkine & Nydegger, 1982). The alternative pathway is activated by certain substances without their prior sensitization with antibodies. It is claimed that in the presence of Mg^{2+} a "priming" C3 convertase, C3,Bb , is formed and that C3 cleavage occurs continuously, and independently of activating substances (Fearon & Austen, 1975a; Pangburn & Müller-Eberhard, 1980). The proteolytic site of the Bb subunit cleaves C3 into C3a and a larger C3b fragment which is bound covalently to cell surfaces and certain substances (Law & Levine, 1977; Law et al., 1980b). Surface-bound C3b binds B in a Mg^{2+} -dependent reaction to form a complex C3b,B (Vogt et al., 1977). B complexed with C3b is cleaved by D, and the alternative pathway amplification convertase C3b,Bb is formed (Fearon et al., 1973; Lesavre & Müller-Eberhard, 1978).

Amplification of C3 activation is partly controlled by spontaneous decay of C3b,Bb (Fearon et al., 1976). In addition, three proteins, P, H, and I are involved in the regulation of the amplification loop. P enhances the activity of the C3 convertase by binding to C3b, thereby stabilizing the complex with Bb (Fearon & Austen, 1975b). H binds to the C3b subunit, blocks uptake of B, accelerates dissociation of Bb, and makes C3b susceptible to cleavage by factor I (Ruddy & Austen, 1971;

Weiler et al., 1976; Pangburn et al., 1977; Whaley & Thompson, 1978). No specific inhibitor to the active enzyme D is known. Lack of the substrate B in complex with C3b is limiting for this step in the reaction sequence (Lesavre & Müller-Eberhard, 1978). The relative affinity of bound C3b for B and H determines the activating properties of a cell surface or of a particle to which C3b is bound. The affinity of C3b fixed to a "non-activator" surface is almost 100 times greater for H than for B, while C3b on a surface that activates the alternative pathway has approximately the same affinity for both B and H. (Pangburn & Müller-Eberhard, 1978; Kazatchkine et al., 1979). A constituent of the cell surface of importance for the affinity between bound C3b and H is sialic acid (Kazatchkine et al., 1979), but human complement is not able to lyse homologous erythrocytes from which sialic acid is removed. This has been attributed to the C3b receptor (CR1), which functionally resembles H in that it dissociates Bb from preformed convertase and facilitates cleavage of C3b by I (Fearon, 1980; Ross et al., 1982).

Interactions between antibodies or classical pathway factors and alternative pathway activation have been observed - for example, human IgG antibodies have been shown to enhance the activation (Polhill et al., 1978; Schenkein and Ruddy, 1980).

The membrane attack complex (MAC) (cf. Boyle & Borsos, 1980; Esser, 1981). MAC is formed from the terminal components C5b, C6, C7, C8 and C9. The convertases $\overline{C4b,2a}$ and $\overline{C3b,Bb}$ or $\overline{C3b,BbP}$, together with additional C3b molecules, acquire C5 convertase activity, splitting C5 into two fragments, C5a and C5b (Vogt et al., 1978; Insenman et al., 1980). C5b forms a complex with C6 and C7. The C5b-7 complex acquires a membrane binding site within the C5 molecule. MAC is formed by the combination of one molecule, C8, with the C5b-7 complex, followed by combination of C9 molecules with the C5b-8 complex (Manni & Müller-Eberhard, 1969; Kolb & Müller-Eberhard, 1974). The attachment of C5b-7 to a membrane is competitively inhibited by the S-protein, which binds to the complex (Podack et al., 1978). Stable fluid phase C5b6 complex can initiate MAC, together with C7, C8 and C9, a phenomenon known as "reactive lysis" (Thompson & Lachmann,

1970; Lachmann & Thompson, 1970). Some controversy exists about the precise mechanism of the membrane damage (cf. Esser, 1981). When lysed, target cells demonstrate characteristic electron microscope membrane lesions (Humphrey & Dourmashkin, 1969).

BIOLOGICAL ACTIVITIES OF COMPLEMENT

During activation, cleavage fragments of complement components are formed which serve as ligands for specific receptors on a variety of cell types. Immune adherence is mediated mainly by the covalently bound complement components C3b and C4b, the latter being less efficient than C3b (Cooper, 1969). The immune adherence to phagocytic cells is the basis of opsonization in the presence of complement.

A possibly important function of complement in immune complex diseases is the solubilization of immune complexes in fresh serum, a process that is mediated mainly by activation of the alternative pathway as reviewed by Takahashi et al., (1980).

C3a and C5a are "anaphylatoxins" - i.e., their binding to receptors on mast cells and basophils results in the release of histamine (cf. Hugli & Müller-Eberhard, 1978). The anaphylatoxin activity is abolished by the action of the anaphylatoxin inactivator (Bokisch & Müller-Eberhard, 1970). C5a has a chemotactic effect on polymorphonuclear leukocytes (Chenoweth & Hugli, 1978). Aggregation of granulocytes by C5a has been ascribed a role in shock states (Jacob, 1981).

A kinin-like fragment derived from C2 has been proposed as the principal mediator of hereditary angioedema (Donaldson et al., 1977b).

Receptors for complement components or fragments present on various cell types have been extensively studied in recent years (cf. Ross, 1982; Sundsmo, 1982; Fearon & Wong, 1983).

BIOSYNTHESIS AND COMPLEMENT METABOLISM (cf. Colton, 1976; Fey & Colton 1981, Lachmann & Peters, 1982)

From in vivo studies it has been shown that at least the components C3, C6, C8 and B are mainly synthesized in the liver (Alper et al., 1980). C1 is synthesized in intestinal epithelial cells (Morris et al., 1978). It has been shown by in vitro studies that macrophages are capable of synthesizing the majority of the complement components. Some of the complement proteins - such as C3, C4, C1s and B - behave like acute phase reactants in inflammation (Schutte et al., 1975).

From studies with radioactive-iodine-labeled components, it has been shown that C1q, C4, C3, B, C5 and H are all normally metabolized with fractional catabolic rates of about 2 % of the intra-vascular pool/hour (cf. Lachmann & Peters, 1982). As judged from studies of patients with reduced glomerular filtration rate, the catabolism of D, like other low molecular weight proteins is mainly determined by renal function (Sturfelt et al., 1984).

GENETICS AND INHERITED ABNORMALITIES OF COMPLEMENT COMPONENTS (cf. Agnello, 1978; Hauptmann, 1979; Glass et al., 1983).

The genes that code for C2, C4 and B are located within the major histocompatibility complex close to the HLA-B locus. Like most other plasma proteins, the complement proteins are subject to allelic variation.

Genetic deficiencies have been reported for all the complement components participating in complement activation, with the exception of B and D. C2 deficiency is the most common one, with a possible incidence of about 1/10,000. About 50 % of those with C2 deficiency and some of those deficient in factors belonging to the terminal sequence remain unaffected by disease connected with the deficiency.

Homozygous deficiency of C1 subcomponents, C2 or C4, is associated with immune complex diseases such as systemic lupus

erythematosus. Hereditary angioedema results from a heterozygous deficiency of $C1$ IA, transmitted as an autosomal dominant trait. $C4$ and $C2$ are persistently low in HAE patients. An association with immune complex disease has also been suggested (Donaldson et al., 1977a).

Severe bacterial infections characteristically occur in conjunction with homozygous $C3$ deficiency. This is also true of secondary $C3$ deficiency with very low $C3$ concentrations, such as in factor I deficiency, and in some patients with $C3$ nephritic factor (Agnello, 1978).

A number of individuals deficient in the late acting components, $C5$ to $C8$, are affected of Neisseria infections, either recurrent meningococcal infections of disseminated gonococcal infections. Deficiency of P was recently reported in a family with fulminant meningococcal infections (Sjöholm et al., 1982).

ACQUIRED COMPLEMENT ABERRATIONS AND DISEASE

There is evidence for complement activation in many diseases, most of which are associated with high concentrations of immune complexes or the presence of activators of the alternative pathway. Acquired deficiencies of certain components may be seen, reflecting hypercatabolism as a result of activation.

Low serum concentrations of classical pathway components may be a prominent feature in immune complex diseases such as systemic lupus erythematosus, and in certain forms of immune hemolytic anemia.

Alterations of serum complement are to be found in certain renal diseases. In poststreptococcal glomerulonephritis, for example, decreased concentrations of both classical and alternative pathway components are seen, although alternative pathway activation would appear to predominate. The complement concentrations in serum from patients with membranoproliferative glomerulonephritis indicate alternative pathway activation. Some sera contain an IgG auto-antibody, termed $C3$ nephritic factor, that is specific for $C3b, Bb$. Binding of the antibody to circulating $C3b, Bb$ results in stabilization of the enzyme and

pronounced C3 cleavage (cf. Lachmann & Peters, 1982).

In patients with Gram-negative bacteremia, low serum concentrations of C3 and evidence of alternative pathway activation have been reported (McCabe, 1973).

A relationship between acquired C1 IA deficiency and neoplastic, often lymphoproliferative, disorders has been recognized (cf. Gelfand et al., 1979). Markedly low serum levels of C1q distinguish the complement profile from that of HAE.

METHODS OF MEASURING COMPLEMENT

Complement can be measured by functional assays for the overall activity of the classical and alternative pathways, or by measurement of the individual components by functional or immunochemical assays (cf. Lachmann & Hobart, 1978). The functional assay most frequently used is the CH50 test (Mayer, 1971), a test yielding information about the integrity of the classical complement system. Although functional tests for the alternative pathway have been described, based on the incubation of serum with activators, none of them has so far been widely used (Götze & Müller-Eberhard, 1971; Forsgren et al., 1975; Soothill & Harvey, 1977; Fong & Renaud, 1978; Lachmann & Hobart 1978; Nydegger et al., 1978; Gewurz et al., 1982).

Specific functional assays, usually based on hemolysis, have been developed for all components in both pathways (cf. Lachmann & Hobart, 1978). These assays are suitable for use only in highly specialized laboratories, immunochemical assays being most frequently used for routine work. In disease, complement activation may lead to manifest hypocomplementemia. Thus, low levels of C1q, C4, C2 and C3 indicate activation of the classical pathway. Alternative pathway activation is characterized by low concentrations of C3, B and/or P together with normal concentrations of C1, C4 and C2.

Detection of activation products, in the form of fragments or complexes of complement components in clinical specimens, will yield further information. Thus, even in the absence of hypocomplementemia, C1 activation may be detected by measuring

$C1r-C1s-C1$ IA complexes - either by electroimmunoassay (Laurell et al., 1979), radioimmunoassay (Hack et al., 1981), or by ELISA (Harpel & Cooper, 1982). Other methods of assessing classical pathway activation are by measuring C4d (Milgrom et al., 1980) or by analyzing C2 cleavage products by crossed immunoelectrophoresis (Sturfelt & Sjöholm, 1983). The use of the ELISA technique in measuring alternative pathway activation, based on the presence of $C3b,Bb,P$ and $C3b,P$ complexes, was recently described (Mayes et al., 1983). The C3 fragment C3d can be measured by electroimmunoassay (Brandslund et al., 1981; Johnson, 1984).

Radioimmunoassays have been developed for measuring the anaphylatoxins, C3a, C4a and C5a (Hugli & Chenoweth 1980; Gorski, 1981).

REUMATOID FACTORS

Waalder (1940) and Rose et al., (1948) first demonstrated that sheep red blood cells, sensitized with subagglutinating amounts of rabbit antibody, were agglutinated by most rheumatoid arthritis (RA) sera. These sera contain anti-immunoglobulins, termed rheumatoid factors (RF), which can be of IgM, IgG or IgA class.

RF react with the Fc-part of IgG. The heterogeneity of RF antibody activity has been demonstrated by reactivity with several IgG determinants in IgG of different origin. The various specificities of RF have been reviewed by Johnson & Faulk (1976).

Between 60-80 % of RA patients, and an even greater proportion of patients with Sjögrens syndrome express serum RF activity when conventional assays are used. RF have also been observed in diseases other than connective tissue diseases - subacute bacterial endocarditis, for example, or chronic liver disease and some tropical infections (for ref. see Hughes, 1977).

Increased amounts of immune complexes are found in serum from patients with RA (Kunkel et al, 1961; Halla et al., 1979).

RF are frequently present in these circulating immune complexes (Pope et al., 1975; Jones et al., 1980; Gabriel & Hadler 1981), and the amount of immune complexes has been shown to be related to the severity of RA (Nydegger et al., 1977; Levo et al., 1981; McDougal et al., 1982).

Various stimuli may induce RF synthesis. Non-specific induction has been demonstrated by B cell activators such as Epstein-Barr virus, pokeweed mitogen and bacterial polysaccharides. RF synthesis has been suggested to be the result of specific induction of B cells by conformationally altered IgG (Johnson et al., 1975), and its induction by denatured IgG has also been reported (Pisko et al., 1982).

The routine methods most frequently used in measuring RF are the Waaler-Rose test and the latex fixation test. These tests, both based on agglutination, are best suited to detect RF of IgM class. To allow separate determination of RF of various immunoglobulin classes several quantitative tests have been developed, most of which are based on the principle of binding RF to insolubilized immunoglobulins, followed by detection by the addition of specific indicator antibodies.

INTERACTIONS BETWEEN RHEUMATOID FACTORS AND COMPLEMENT

The possibility that RF can activate complement in vivo has been studied for many years. Since the initial observations of lowered complement concentrations in synovial fluids in conjunction with classical sero-positive RA (Hedberg, 1963; Pekin & Zvaifler, 1964), many other studies have suggested interaction between RF and complement. Evidence of in vitro complement activation by polyclonal IgM-RF, via the classical pathway, was given by Tanimoto et al., (1975), using a hemolytic assay system. Classical pathway activation by IgM-RF and IgG-RF has recently been demonstrated, using a radioimmunoassay for C4 (Sabharwal et al., 1982).

Complement activation products have been detected in RA sera (Versey et al., 1973; Lambert et al., 1975; Nydegger et al., 1977; Perrin et al., 1977; Berglund et al., 1980). Meta-

bolic turnover studies almost invariably reveal hypercatabolism of C3 (Weinstein et al., 1972; Ruddy et al., 1975). The predominance of classical pathway activation has been shown by the increased catabolism of C4, which correlated to the amount of RF present (Kaplan et al., 1980). However, complement concentrations in RA sera usually lie within the normal range, or they are elevated (Vaughan et al., 1951; Berglund et al., 1980), which is probably to be explained by the increased synthesis that is an integral part of the inflammatory reaction.

THE PRESENT INVESTIGATION

The aim of the investigation has been to develop methods of estimating the complement system that are suitable in clinical laboratory work.

1. A hemolysis in gel (HIG) procedure for detecting various complement deficiency states is described. Hemolysis of sensitized sheep erythrocytes was used for estimating classical pathway function. The integrity of the alternative pathway was assessed by lysis of guinea pig erythrocytes.

Factors causing aberrant patterns of impaired hemolysis in the test for the classical pathway, unrelated to complement disorders, were further analyzed in sera from patients with rheumatoid arthritis (RA), and in sera from IgA deficient individuals.

2. Rheumatoid factors (RF) capable of activating complement were studied by hemolytic assays. Concentrations of complement activating RF in RA were investigated with regard to C1 activation in the circulation, and to C1q binding immune complexes. An enzyme linked immunosorbent assay (ELISA) was developed for measuring class-specific RF.

3. Reference areas for newborns and fullterm pregnant women were given for 16 proteins belonging to the complement system. An immunochemical method for measuring of factor D of the alternative pathway was developed and compared with functional assays. Reference areas for D in healthy adults were established.

1. SCREENING FOR COMPLEMENT DEFICIENCIES BY HIG (I, II, III)

Methods and Results

Technical procedures.

Lachmann and Hobart (1978) have described the general procedure for using the HIG technique in complement studies. In the screening test dependent on classical pathway activation (I), E sensitized with rabbit anti-sheep erythrocyte antibody (EA) were used. E sensitized with IgG (EAG) or IgM (EAM) antibodies were used in some analyses (III). The lowest non-limiting lytic dose of antibodies determined by amboceptor titration was used for sensitization (Rapp & Borsos, 1970). EA at 1 % (v/v) were incorporated in 0.6 % agarose (Agarose (ME), Marine Colloids Inc), in veronal-buffered saline with 0.15 mmol/l Ca^{2+} and 0.5 mmol/l Mg^{2+} (VBS⁺⁺). Five μl of undiluted serum or plasma were added to wells of 3.0 mm diameter. Incubation was carried out in a humid atmosphere at 4 °C for 18 h and then a 37 °C for 2 - 3 h.

The procedure for the alternative pathway assay was the same as that described above. GpE at 1 % (v/v) and VBS with 10 mmol/l Mg^{2+} and 10 mmol/l EGTA was used. The plates were used the same day they were prepared to avoid disturbing "background" lysis, and kept at 4 °C during application of the samples.

Either serum or plasma could be used. Storage of normal serum at room temperature for two days did not alter the patterns in either assay. In neither normal nor pathological serum samples were lytic patterns appreciably affected by freezing at -80 °C and thawing up to five times.

The HIG assay, for detecting factors inhibitory to activation of the classical pathway was performed with agarose plates containing 1 % (v/v) of either EAG or EAM (II, III). Samples were heated at 56 °C for 30 min before being applied to wells. After incubation at 4 °C for 18 h, the gel was supplied with complement and incubated for another 2 h at 4 °C, and finally lysis was induced by incubation at 37 °C for 2 - 3 h. Guinea pig

serum (GPS) was generally used as complement source, though human serum was equally efficient. GPS was absorbed with E to avoid disturbing lysis due to possible anti-E antibodies. It was found most convenient to add GPS in filter papers placed on top of the gel.

HIG screening assays in normal adults, full-term women and newborns (Table 2).

With the classical pathway test, full lysis was seen in about 95 % of sera from normal blood donors (I). Sera from full-term women in labour gave full lysis in 86 % and sera from newborns were normal in 87 % (unpublished).

Table 2. Results of the HIG screening assays for the classical pathway (EA) and for the alternative pathway (GpE), with sera from healthy adults, full-term pregnant women and newborns.

	Lysis of EA (per cent of sera)			Lysis of GpE (per cent of sera)		
	Normal	¹ Impaired	No	Normal	¹ Impaired	No
Healthy adults (n=105)	97	3	0	94	5	1
Full-term pregnant women (n=100)	86	12	2	99	1	0
Newborns (n=100)	87	13	0	42	40	18

¹Partial lysis, zones of unlysed cells within the lytic area or small area of lysis.

In the alternative pathway test nearly all adult sera showed full lysis, whereas most sera from newborns showed only partial lysis or none at all. Low concentrations of one or more of the complement components measured (VII), with low C6, C8, or both, being particularly common, probably accounted for the reduced hemolytic capacity of neonatal sera.

In maternal sera, reduced hemolysis was not related to low concentrations of any of the 16 complement components measured (VII). No factors inhibitory to immune hemolysis were detected by HIG (unpublished). Complement-mediated hemolysis is reported to be inhibited by pregnancy-associated protein-A (Bischof, 1981). The concentration of this protein in the maternal sera was determined by electroimmunoassay (Laurell, 1972), using a commercial antiserum (Dakopatts, Denmark). The samples were diluted 1/4 in 75 mmol/l barbitol-HCl buffer, pH 8.6, containing Na_2EDTA (2 mmol/l), the buffer also used in running the electrophoresis. The values obtained varied widely, from < 25 up to 720 % of a serum reference pool, but were unrelated to the outcome in the HIG assays (unpublished).

Sera with selective deficiencies of complement components (I).

Sera from patients with complete deficiencies of C1q, C2 and C4 produced full lysis only of GpE. There was no lysis of EA in C2 and C4 deficient sera, whereas two of three C1q deficient sera produced a peripheral zone of partial lysis, and the third no lysis at all. C3 deficient serum produced very faint lysis of EA and none of GpE. C8 deficient serum showed no lytic activity, P deficient serum was normal in the classical pathway test and produced partial or no lysis in the alternative pathway test.

Reconstitution of the deficient sera before analysis resulted in normal hemolysis in both assays, with the exception of one C1q deficient serum (I); later, however, it was found that reconstitution of another sample from the C1q deficient patient (Loos et al., 1980) resulted in full lytic activity in the HIG assay (unpublished).

Sera with deficiencies of control proteins (I).

Serum from a patient with HAE produced no lysis of EA, but normal lysis of GpE, while serum from a patient with homozygous deficiency of factor I produced a small area of lysis in the assay with EA, but no lysis of GpE.

Diseases with pronounced complement activation (I).

Of sera from two patients with chronic urticaria studied, one - with C1q less than 5 % and high amounts of C1r-C1s complexes - failed to lyse EA, and the other with a normal C1q concentration but a high concentration of C1r-C1s-C1 IA complexes - produced a small lytic area with EA. Both gave full lysis of GpE. Sera from two patients with acute poststreptococcal glomerulonephritis and one with membranoproliferative glomerulonephritis, all with low concentration of C3, caused small areas of partial lysis of EA but no lysis of GpE.

Sero-positive rheumatoid arthritis.

Among the various pathological sera which gave impaired hemolysis in the classical pathway test without obvious relationship to complement disturbances were many samples from patients with RA (I). To examine the influence of rheumatoid factors (RF) on the outcome in the HIG assays, 37 sera from patients with sero-positive RA (ARA criteria) were studied (II). Nine of the sera showed an abnormal pattern in the HIG test with EA, but all were normal in the test for the alternative pathway. Serum concentrations of factors C1q, C1s, C4 and C3 were not reduced.

The aberrant lytic patterns were zones of unlysed cells, either adjacent to the well, or within the lytic area. Absorption with E resulted in elimination of the inner lytic zone. Using a two-step HIG assay, all sera were shown to inhibit lysis of EAG (II). The inhibition areas were correlated to the concentrations of agglutinating RF ($r = 0.80$, $p < 0.001$, Spearman rank correlation test). Absorption of serum with rabbit IgG coupled to Sepharose 4B reduced the inhibition capacity, and affinity purified RF also inhibited immune hemolysis in the

assay. In 26 of the 37 sera, increased concentrations of circulating immune complexes (CIC) were found in the C1q deviation test. Only one serum, with >4000 mg IgG equivalents/l, inhibited lysis of EAM. No clear relationship was noted between the inhibition of lysis of EAG and the concentrations of CIC.

IgA-deficient sera (III).

This part of the study was prompted by the aberrant pattern of impaired hemolysis observed in the classical pathway test with several IgA deficient sera. Ten of 17 IgA deficient sera produced impaired or no lysis of EA, while all the sera tested were normal in the alternative pathway test. Sera showing inhibition in HIG tests, and those showing full lysis, were not distinguishable from each other with regard to hemolytic activity, as measured by CH50. The values of the individual complement factors were mainly normal, and no relationship to abnormal lytic patterns was observed. The impaired lysis remained when EAM was used in the classical pathway test, but not when EAG was used.

The inhibitory sera did not agglutinate EAG, but agglutinated EAM with titers that were closely correlated to inhibition areas obtained in a HIG assay for the detection of inhibitory factors ($r = 0.74$, $p < 0.01$, Spearman rank correlation test). Absorption with EAM resulted in normal lysis. In a combination of agarose electrophoresis and HIG, the inhibiting factor was shown to be located in the γ -region. Absorption with protein A, performed with one serum, removed this factor, and its eluate was inhibitory in the HIG assay. After incubation of EAM with the same serum, an uptake of IgG antibodies on the erythrocytes was demonstrated, using ^{125}I anti-human IgG antibodies.

The inhibition phenomenon was as frequent in IgA deficient sera from clinically healthy individuals as in those with significant disease. No relation was found to sex or age.

Discussion

The HIG technique was originally introduced by Martin et al., (1962) and Milgrom & Millers (1963) as a complement fixation test. In the present study, two parallel HIG assays were used to assess the functional activity of complement in serum and plasma. In the assay using EA, the classical pathway is activated through the reaction of C1 with cell bound antibody. GpE are considered to be lysed by late acting components in the complement sequence, fluid phase alternative pathway activation being due to Mg^{2+} and agarose (Lachmann & Hobart, 1978). EGTA chelates Ca^{2+} , thereby preventing classical pathway activation.

Together, the two assays distinguish between defects of the classical, alternative or terminal pathway (I). Reconstitution of the deficient sera with purified components provided normal lytic patterns. Hypocomplementemia, secondary to C1 IA and factor I deficiency, was also demonstrated. In routine diagnostic work since 1980, the pattern of partial or absent lysis in the classical pathway test, and of full lysis in the alternative pathway test, has been consistently observed in repeated samples from 18 patients with well-documented HAE (unpublished observation).

Most healthy blood donor sera produced homogenous areas of clear hemolysis, 4 % of which were aberrant (I). Of the neonatal sera, the majority were normal in the classical pathway test, whereas less than half of them produced full lysis of GpE, which could be accounted for by low serum concentrations of one or several complement factors. The frequent occurrence of subnormal alternative pathway activity in newborns has been reported by others (Adamkin et al., 1978; Strunk et al., 1979; Johnston et al., 1979).

The fact that 14 % of sera from the full-term mothers failed to produce full lysis of EA, could not be ascribed to low complement concentrations, or to such inhibitory factors as were shown to be anti-immunoglobulins (II, III). Pregnancy-associated plasma proteins or changes in concentrations of normal plasma

proteins might affect the lysis of EA. However, pregnancy-associated plasma protein-A, which is reported to inhibit complement-mediated hemolysis when present in the concentrations occurring in sera from pregnant women (Bischof, 1981), was not responsible for the impaired hemolysis observed here.

Various pathological sera, including those from patients with RA, showed patterns of impaired lysis of EA, that could not be explained by hypocomplementemia (I). A zone of unlysed cells was present within the lytic area. Most sera produced a narrow lytic zone adjacent to the well, that appeared to be dependent on the presence of Forssman antibodies. (III). Although Thompson and Rowe (1967) reported that rarely occurring antibodies to E can inhibit immune hemolysis, such antibodies could not have been responsible in the sera studied here, since absorption with E did not remove the inhibition zone (II, III). The pattern of a ring of unlysed cells within the lytic area was shown to be caused by the action of anti-immunoglobulins (II, III).

IgA deficient sera were shown to contain antibodies to rabbit IgM in high frequency, which inhibited lysis of EA in the HIG test (III). In serum from an IgA deficient patient, the inhibiting antibodies reacted with protein A and ^{125}I anti-human IgG, demonstrating that the inhibitory antibodies were of IgG class. A similar observation has been reported by Johnson et al. (1976). Antibodies to human IgM (Wells et al., 1972), and bovine IgM (Huntley et al., 1971; Leikola & Vyas, 1971; Tomasi & Katz, 1971) have been reported in IgA deficiency. It is not known what mechanism causes the development of antibodies to IgM from various species in IgA deficient individuals. Anti-rabbit IgM antibodies were unrelated to the patient's symptoms (III). Such antibodies are not found in RA, but may cause "false-positive" Waaler-Rose test when unfractionated antiserum is used in the sensitization of E (Truedsson & Sturfelt, 1983).

Twenty-four per cent of sera from patients with RF-positive RA produced impaired lysis of EA (II). The RA sera consistently inhibited lysis of EAG in a two-step HIG assay with GPS as the complement source and so did RF purified by affinity chromatography. From these findings and the close correlation between inhibition areas and RF agglutination titers, it was concluded

that RF was the main cause of inhibited immune hemolysis. This agrees with findings of Romeyn et al., (1975) who reported inhibition by classical RF in a similar assay.

The mechanism by which anti-immunoglobulins inhibit hemolysis in agarose gel is not clear, but might involve either blocking effects or hemolytically inefficient complement activation on the surface of antibody coated cells. It should be pointed out that inhibitory effects of anti-immunoglobulins on immune hemolysis were not demonstrable in diluted assay systems, such as the CH50 test (III). The findings suggest that anti-immunoglobulins can modify complement activation under conditions that might possibly apply in vivo.

Although the possibility can not be excluded that soluble immune complexes in pathological sera contribute to inhibition of immune hemolysis, with one exception the inhibition of hemolysis by the RA sera tested here could not be ascribed to circulating immune complexes (II).

2. COMPLEMENT ACTIVATION BY RHEUMATOID FACTORS (II, IV)

Methods and Results

Measurement of complement activating RF (IV).

Reduced and alkylated IgG, with very low complement activating capacity but with preserved antigenic properties, have been used for the sensitization of cells in hemolytic assays for RF (Tanimoto et al., 1975). In the present study, such cells were utilized in a HIG assay for complement activating RF. Reduced and alkylated rabbit IgG antibodies were prepared according to Johnson and Hoffmann (1981). Agarose plates were prepared containing 0.75 % (v/v) E, sensitized with an appropriate dose of antibodies. Seven μ l of test samples, heated at 56 °C for 30 min and absorbed with E, were applied to 3.0 mm diameter wells in the gel. After diffusion at 4 °C for 64 h, GPS was supplied to the gel and the plate incubated at 37 °C for 2 - 3 h. The diameters of the lytic zones were measured and the results expressed in arbitrary units, defined with an RF reference.

Sera from 16 patients with RA (ARA criteria) (Berglund et al., 1980) were studied. In general, full lysis was observed with the sera. Dilutions of patient sera gave titration curves parallel to those obtained with the reference serum and with affinity purified RF (IV).

Values obtained with the RA sera in the hemolytic tube assay (Tanimoto et al., 1975) were closely correlated with lytic areas in the HIG assay for RF ($r=0.89$, Spearman rank correlation test), and with inhibition areas in the HIG inhibition assay (II) ($r=0.84$). The zone areas in the two different HIG assays were also closely correlated ($r=0.81$).

Measurement of class-specific RF (IV).

An enzyme linked immunosorbent assay (ELISA) was developed. Human serum albumin was adsorbed to the surface of a microtiter plate. An IgG fraction of anti-human serum albumin antibodies

was then added. Serum samples containing RF were applied in dilutions of 1/100 or more. The RF bound to the rabbit IgG antibodies were detected by the addition of alkaline-phosphatase conjugated $F(ab')_2$ fragments of rabbit antibodies specific for α , γ or μ chains. Each serum was tested in triplicate wells, of which one was a control coated with serum albumin alone. In each plate an RF reference was included. The results were given in arbitrary units calculated from the absorbance values for each sample and that of the reference.

With different sera, fairly parallel titration curves for both IgG-RF, IgA-RF and IgM-RF were obtained. Addition of purified C1q did not influence the results. The reproducibility of the ELISA was satisfactory. RF of the three immunoglobulin classes were detected in all the 16 RA sera except one IgA deficient serum.

Relationship between complement activating RF, class-specific RF and disease activity (IV).

In sera from the 16 RA patients, the concentrations of complement activating RF, as estimated by HIG, were closely correlated with RF of IgM class ($r=0.86$, Spearman rank correlation test), but not with IgG-RF or IgA-RF. Disease activity, as reflected by a synovitis index (Berglund et al., 1980), was not correlated with RF measured with any of the assays. After administration of podophyllotoxin derivatives for 6 months, the concentrations of RF were markedly decreased, as measured by the different assays ($p<0.001$). From the start of treatment, the decreases of IgM, IgG and IgA were less pronounced than those of the corresponding class-specific RF.

Relation between complement activating RF, C1 activation and C1q binding immune complexes (IV).

Measurement of $C\bar{1}r-C\bar{1}s-C\bar{1}$ IA complexes by electroimmunoassay (Laurell et al., 1979) gave high values, previously shown to correlate with disease activity expressed as a synovitis index (Berglund et al., 1980). Complement activating RF were not significantly correlated with the concentration of $C\bar{1}r-C\bar{1}s-C\bar{1}$ IA complexes ($r=0.38$, Spearman rank correlation test), nor to the

C4 concentrations in serum ($r=-0.22$). C1q binding immune complexes were present in nearly all sera. The amounts of CIC determined by the C1q binding assay were correlated to complement activating RF ($r=0.88$), but CIC measured with C1q deviation test were not ($r=0.38$). The occurrence of partial lysis in the HIG assay for RF seen with some sera were not related to CIC concentrations.

Discussion

Direct measurement of RF with complement activating activity could be accomplished by a simple HIG assay, reduced and alkylated IgG being used to sensitize of the cells (IV). RF were also able to inhibit immune hemolysis when cells were coated with intact rabbit antibodies (II). The close correlation between the two different HIG assays and the original hemolytic tube assay for RF (Tanimoto et al., 1975) suggest that the same population of RF molecules was responsible. Thus, in one HIG assay (II) RF inhibit immune hemolysis, but in the other assay with reduced and alkylated IgG on the cells (IV), complement activation is necessary for the lytic reaction. RF of IgA class, incapable of activating complement, were probably of limited consequence in the assays, since IgA deficient serum also inhibited lysis of cells sensitized with intact IgG (IV). Since factors other than RF, such as CIC, may inhibit immune hemolysis (II), the lytic HIG assay for complement activating RF should be more specific. Hemolytically inefficient complement activation on the cell surface is a possible explanation for the ability of RF to inhibit lysis of erythrocytes coated with intact IgG antibodies.

Class-specific RF were measured with an ELISA. $F(ab')_2$ or $F(ab)$ fragments of the indicator antibodies must be used in such assays (Tarkowski et al., 1983). As shown by the titration curves, blocking of binding sites for RF of one immunoglobulin class by RF of another class was not observed under the conditions pertaining here.

The findings suggested that complement activation in the

assays was largely dependent on IgM-RF. This is in accordance with the findings of Sabharwal et al., (1982), who reported a much more efficient complement activating capacity for IgM-RF than for IgG-RF.

In the RA patients studied, concentrations of $\text{C}\bar{\text{I}}\text{r}-\text{C}\bar{\text{I}}\text{s}-\text{C}\bar{\text{I}}$ IA complexes, as a measure of C1 activation, correlated to disease activity (Berglund et al., 1980). The present findings indicated that the increased concentrations were not primarily due to circulating RF. However, possible C1 activation through RF containing complexes that were not directly measured in the assays, can not be excluded. Moreover, antibodies to collagen (Trentham et al., 1982), C reactive protein containing complexes, and granulocyte proteases (Hadler et al., 1979), are other possible C1 activators in RA.

3. MEASUREMENT OF COMPLEMENT COMPONENTS (V, VI, VII)

Methods and Results

Purification of D and production of antiserum (V).

A purification procedure was elaborated by a combination of ion-exchange chromatography and gel filtration. Highly purified D was prepared from plasma with a yield of approximately 30 %. Rabbit antiserum was raised by two injections, of about 0.3 mg purified D emulsified with Freund's adjuvant, given with an interval of three weeks. Double immunodiffusion with unabsorbed antiserum was performed against normal human serum (NHS) and D preparation. Single precipitation lines, visualized using enzyme amplifying technique (Löfberg & Grubb, 1979), were obtained, showing a reaction of identity. Addition of antiserum to NHS abolished the lytic reaction in a HIG assay for D, providing further evidence for the specificity of the antiserum.

Immunochemical measurement of D (V).

Electroimmunoassay was performed according to Laurell (1972), using 75 mmol/l barbital buffer containing either 2 mmol/l EDTA or 2 mmol/l Ca^{2+} in the gel. In preliminary trials, low endosmosis agarose was found most suitable. To obtain distinct immunoprecipitates, 5 % (w/v) polyethylene glycol 6000 (Kebo AB, Stockholm) was added to the gel. The precipitates were stained with horseradish peroxidase-labeled swine antibodies to rabbit immunoglobulins, as described by Löfberg & Grubb (1979) for enzyme amplified single radial immunodiffusion.

When the enzyme amplified electroimmunoassay (EAE) was performed with Ca^{2+} in the buffer, both cathodal and anodal precipitates were obtained with NHS, while only anodal peaks were observed using a buffer containing EDTA. For this reason the buffer containing EDTA was subsequently used in the assay.

With purified D diluted in buffer, lower immunoprecipitates were obtained than when dilutions were made in D-depleted serum (V, VI).

D in normal serum and plasma (V, VI).

The D content in a reference serum pool (148 normal blood donors) was 1.6 mg/l, as assessed by the EAE (V). Measurement of paired serum and plasma samples gave similar or somewhat lower values for plasma samples. Due to the positively skewed D values in normal sera, logarithmic transformation was used in calculating reference areas. The 95 % geometric reference area for healthy adults ($n = 144$) was 75- 143 % of the reference when determined by EAE, and 65 - 171 % when determined by a HIG assay (VI). The mean serum concentration of D was slightly higher for males than for females, the largest difference seen for values obtained with the HIG assay being barely significant ($p < 0.05$).

Comparison of immunochemical and functional methods for measuring D (VI).

Serum values of D in 144 healthy adults, determined by HIG and by EAE, were correlated, $r = 0.52$ ($p < 0.001$). The differences between the values obtained with the two methods were not statistically significant (paired t-test). In the high range, the functional assay gave higher values than the immunochemical method, while in the low range, the relationship was reversed. As estimated with the two methods, these differences, like that between mean values, were not significant. Comparison between D values obtained by EAE in 20 normal sera, and D determined with another functional assay based on hemolysis of rabbit erythrocytes (Lesavre et al., 1979), showed the same relationship (VI).

Variation of D serum concentrations with age (VI, VII).

D values obtained by the EAE for groups of newborns, healthy children (2-10 years), pregnant women, and healthy blood donors (19-61 years), are given in Table 3. The cord samples from newborns contained higher concentrations of D than did the adult

sera, whereas the children had lower serum values. Within the blood donor group, no significant difference was noted between mean D values in the group aged 19-40 years ($n = 72$) and the group aged 40-61 years ($n = 72$) (VI).

Serum concentrations of complement components in newborns and pregnant women (VII).

To establish reliable reference values of complement components in newborns, samples of blood were obtained from 100 healthy full-term women in labour and, after delivery, from their infants. By electroimmunoassay, the complement components C1q, C1r, C1s, C1 IA, C2, P,D, I, H, C6 and C7 were measured in serum, and the factors C4, C3, B, C5 and C8 in EDTA-plasma. Due to a positively skewed distribution of several components (C4, B, D and C7), the values were logarithmically transformed and the geometric means and 95 % geometric confidence intervals calculated.

Table 3. D serum concentrations determined by enzyme amplified electroimmunoassay (EAE) in different groups of healthy individuals. The values are given in per cent of the concentration in a serum reference pool.

	Arith. mean	1 stand. dev.	Geom. mean	Geom. 95 % conf.area
Healthy blood donors (19-61 years, $n = 144$)	105	17	104	75-143
Pregnant women in labour ($n = 100$)	88	17	85	59-125
Healthy children (2-10 years, $n = 29$)	73	9	73	56-95
Newborn infants ($n = 100$)	126	25	124	84-183

In the newborns, all components but D and C7 showed mean values below those of healthy adults. The lowest concentrations, less than 50 % of the adult values, were found for factors P, C6 and C8. Factor D differed from the other proteins measured, in that D showed higher concentrations in the cord samples (124 %) than in the maternal ones (86 %). The C7 values in both cord and maternal samples were high as compared to the adult reference. In the mothers, the majority of components had higher mean values, up to about 150 % of that in the reference.

Discussion

Immunochemical methods for the measurement of complement components in serum are easily performed and show good precision and specificity. Reference values for healthy adults have been established by electroimmunoassay for several complement components (Sjöholm, 1975; Laurell et al, 1978).

In this study, a simple immunochemical assay for factor D was developed (V). Although this protein has previously been measured by a hemolytic assay (Martin et al., 1976; Wilson et al., 1979), a disadvantage with this method is the possible interference of other enzymes which may be present in pathological sera. Thus, it has been reported that enzymes such as trypsin (Brade et al., 1974), and kallikrein (DiScipio, 1982), can replace D in the cleavage of factor B. The presence of proteases in pathological sera might result in spuriously high D values.

In order to prepare specific antiserum to D, a purification procedure was elaborated (V). A combination of ion-exchange chromatography and gel filtration was used, as outlined by Volanakis et al., (1977). Highly purified D was obtained and the yield was approximately 30 %, which is higher than with other reported methods (Volanakis et al., 1977; Davis et al., 1979; Lesavre et al., 1979; DiScipio, 1981).

Measurement of D concentration in serum by electroimmunoassay was made possible by introduction an enzyme amplified

staining procedure as first described by Alpert et al., (1974). The sensitivity of this technique is equal to that of autoradiography (Kjaervig Broe & Ingild, 1983). Factor D could be detected at 50 $\mu\text{g/l}$, which is far below the normal serum concentration (V). The amplifying step also makes it possible to economize with the specific antiserum in the gel.

To obtain distinct immunoprecipitates, it was necessary to add polyethylene glycol 6000 to the agarose gel. The ability of this polymer to enhance antigen-antibody precipitation is well documented (Hellsing, 1973).

The cathodal and anodal precipitation peaks observed when a buffer containing Ca^{2+} was used confirm the observation that factor D has γ -mobility in the presence of Ca^{2+} or Mg^{2+} ions, but β -mobility in the presence of EDTA (Davis et al., 1979). The height of peaks obtained with purified D in buffer was lower than with D in D-depleted serum. Binding of D to another serum protein in the presence of EDTA has been proposed as an explanation of this (Konno et al., 1978), adsorption of D to the agarose being another possibility. Purified D together with human serum albumin gave precipitation peaks similar to those obtained with D in serum depleted of D. On the basis of these observations it seems advisable to dilute sera to be measured by EAE in D-depleted serum.

The normal serum concentration of D found in this study was 1.6 mg/l, which is in good agreement with earlier reports (Lesavre & Müller-Eberhard, 1978; Lesavre et al, 1979). D serum concentrations determined by HIG and EAE agreed well, the discrepancies seen not being significant (VI). Thus, neither presence of non-functional D protein, nor other factors expressing D activity in normal sera, was indicated by the results.

Reference values were established for 16 complement components in the newborn (VII). Where an inherited disorder within the complement system is suspected, or in newborn infants with uncharacteristic signs of illness, it may be important to measure single complement factors. Previous reports have usually been based on studies of small groups applying various methods and reference values (cf. Johnston et al., 1979).

With the exception of factor D and C7, the mean values for newborns were lower than for their mothers and for healthy blood donors. The lowest serum concentrations were observed for the late acting components C6 and C8, and for the alternative pathway factors, P and B. The mean value for C7 was approximately twice that reported by Adolfini & Beck (1975), who used a functional assay.

The mean D serum concentration in the newborns was higher than those in the adult groups. Findings of increased plasma values of the low molecular weight proteins, β_2m and γ -trace in newborn infants have been reported (Aperia & Broberger, 1979; Löfberg et al., 1980), which demonstrate high production in relation to their glomerular filtration rate (Löfberg et al., 1980). This might also be true for the low molecular weight protein factor D. Low molecular weight proteins are catabolized by the kidneys (Manuel et al., 1975). In patients with lupus nephritis or polycystic kidney disease the serum concentrations of D, like these of β_2m and γ -trace, are correlated to glomerular filtration rates (Sturfelt et al., 1984).

The majority of the complement components were present in higher concentrations in the maternal samples than in the adult reference material. Opferkuch et al., (1978) found no such difference. In their study it was not stated during which period of pregnancy the samples were collected, but in the present study all samples were drawn from full-term women.

CONCLUDING REMARKS

The need for screening, to rule out complement deficiencies is obvious, and has been stressed earlier by other authors (Gewurz et al., 1982; Weinstein et al., 1983; Merino et al., 1983). The HIG screening procedure (I) consistently discloses selective complement deficiencies and sera with pronounced complement consumption. The combination of one classical pathway assay and another assay for the alternative pathway allows defects of both pathways to be distinguished. Even patterns suggesting the presence of anti-immunoglobulins, or possibly circulating immune complexes can be distinguished (II, III). The value of the screening method is limited in newborns, since less than 50 % of them had normal alternative pathway function.

To estimate individual components, reliable reference values for different age groups are necessary, as is illustrated by the differences in serum concentrations of several components between newborns and adults (VII). Reference values for several complement factors in children of different age groups are still not available. Electroimmunoassay is a suitable method for measurement and by enzyme amplified electroimmunoassay, it is even possible to measure factor D in large quantities of clinical material (V, VI).

The assessment of the complement system by determining the serum concentrations of complement components, "the complement profile", has limitations. Although activation results in low values for one or more factors, increased synthesis may disturb the pattern. Furthermore, activation may proceed at a localized site, and not result in any change of the serum concentration of complement.

Further information may be obtained by the detection of activation products such as complexes or fragments of components. This is illustrated in RA, where complement activation products are usually found together with normal serum concentrations of complement. Activation products may be measured by immunoprecipitation techniques, radioimmunoassay or the recently developed ELISA technique.

S U M M A R Y

A combination of two assays based on hemolysis in gel (HIG) were evaluated as a screening procedure for detecting complement deficiency states. For the classical and alternative pathways, respectively, lysis of sensitized sheep erythrocytes (EA) and guinea pig erythrocytes (GpE) were used. With few exceptions (about 4 %), fresh samples from adult blood donors produced homogenous areas of complete hemolysis in both assays. With sera from newborn infants, normal lysis of EA was obtained in 87 % whereas full lysis of GpE only in 42 %. Sera from the full-term mothers produced normal lysis in 86 % of EA, and in 99 % of GpE. Sera from patients with selective deficiencies of complement components were clearly distinguished, as were sera with pronounced complement activation.

Various pathological sera gave patterns of impaired hemolysis in the classical pathway test, that could not be explained by hypocomplementemia. Nine of 37 sera from patients with classical rheumatoid arthritis (RA) produced impaired lysis of EA, while full lysis of GpE was obtained. The inhibition of hemolysis was shown to depend mainly on the presence of rheumatoid factors (RF).

In IgA deficiency patterns of impaired hemolysis in the classical pathway test, together with normal alternative pathway function, were found in 10 of 17 sera. The impaired hemolysis was not related to complement aberrations, but was caused by antibodies to rabbit IgM, the presence of these antibodies being unrelated to age, sex or disease.

Complement activating RF were measured in sera from 16 patients with RA by a simple HIG technique. Measured by enzyme linked immunosorbent assay, IgM-RF, but not IgG-RF or IgA-RF, were closely correlated to complement activating RF. The concentrations of RF were not directly related to C1 activation in the sera. Immune complexes measured by C1q binding assay were closely correlated to complement activating RF and IgM-RF. Treatment of the patients with podophyllotoxin derivatives

clearly reduced the concentrations of circulating RF.

Factor D of the alternative pathway was prepared with a yield of about 30 %, and a monospecific rabbit antiserum was raised. D in serum and plasma was measured by enzyme amplified electro-immunoassay (EAE). Fifty $\mu\text{g/l}$ could be detected. The concentration of D in a normal serum reference pool was 1.6 mg/l, as assessed by this assay. The 95 % geometric confidence areas for D in 144 adult blood donors were 75-143 % of the reference as determined by (EAE), and 66-171 % as determined by a HIG assay. D values obtained with the two assays were correlated ($r = 0.52$, $p < 0.001$). Factor D concentrations were lower in children (2-10 years) than in adults.

Reference values for 16 complement components in 100 newborns and their mothers were determined. By electroimmunoassay, components were measured in serum (C1q, C1r, C1s, C1 $\bar{\text{I}}$ IA, C2, P, D, I, H, C6 and C7), or in EDTA-plasma (C4, C3, B, C5 and C8). In the newborns, the lowest mean values were those recorded for factors P, C6 and C8 (46, 44 and 36 per cent of the adult reference levels, respectively). Only D and C7 showed mean values above those of healthy adults. The maternal levels of C2, C4, C3, B, H, C5 and C6 were 40-60 per cent higher than those of the reference, while C1q, C1 $\bar{\text{I}}$ IA, P and D showed lower mean values.

ACKNOWLEDGEMENTS

I would like to thank the many people who contributed their help and cooperation during the work on these studies. In particular, I wish to express my sincere gratitude to:

Prof. Anna-Brita Laurell, my chief and my mentor, for her encouragement and constructive criticism;

Prof. Rune Grubb and Prof. Lars Kjellén, for providing excellent working facilities;

Ass. prof. Anders Sjöholm and ass. prof Ulf Johnson, for their invaluable help and rewarding cooperation;

Dr. Gunnar Sturfelt, my close friend and colleague, for his inspiring and fruitful cooperation;

Miss Birgitta Ellerström, Miss Gunilla Nordin, Miss Katarina Jönsson, Mrs. Ulla Mårtensson and Mrs. Eva Holmström for their skillful technical assistance;

Mrs. Britt-Marie Karlsson for typing manuscripts, Mr Åke Christensson for taking photographs, and finally, Mr Richard Pybus for his instructive revision of my English.

Financial support was given by the Swedish Medical Research Council (B83-16X-68), the Medical Faculty, University of Lund, Forssmans fond, Greta och Johan Kocks Stiftelser, Riksförbundet mot Reumatism, Thorsten och Elsa Segerfalks Stiftelser, and Alfred Österlunds Stiftelse.

Reproduction of the original papers has been kindly permitted by Acta Pathologica, Microbiologica et Immunologica Scandinavica, Copenhagen and Elsevier Science Publishers B.V., Amsterdam.

R E F E R E N C E S

- Adamkin D, Stitzel A, Urmson J et al.: Activity of the alternative pathway of complement in the newborn infant. *J. Pediatr.* 93: 604, 1978.
- Adolfini M, Beck SE: Human complement C7 and C9 in fetal and newborn sera. *Arch. Dis. Child.* 50:562, 1975.
- Agnello V: Complement deficiency states. *Medicine* 57:1, 1978.
- Allan R, Isliker H: Studies on the complement-binding site of rabbit immunoglobulin G. II. The reaction of rabbit IgG and its fragment with C1q. *Immunochemistry* 11:243, 1974.
- Alper CA, Raum D, Awdeh ZL et al.: Studies of hepatic synthesis in vivo of plasma proteins, including orosomucoid, transferrin, α -antitrypsin, C8 and factor B. *Clin. Immunol. Immunopathol.* 16:84, 1980.
- Alper CA et al.: Nomenclature of the alternative activating pathway of complement. *Bull. Wld. Hlth. Org.* 59:489, 1981.
- Alpert E, Coston R, Perrotto J: Immunoenzymatic assay for alpha-fetoprotein. *Lancet* i:626, 1974.
- Aperia A, Broberger U: Beta-2-microglobulin. An indicator of renal tubular maturation and dysfunction in the newborn. *Acta Pediatr. Scand.* 68:669, 1979.
- Austen KF et al.: Nomenclature of complement. *Bull. Wld. Hlth. Org.* 39:935, 1968.
- Austen KF: The complement system. P 218 in Textbook of Immunology. Eds. B. Benacerraf, ER Unanue. The Williams & Wilkins Company, Baltimore 1979.
- Becker EL: Concerning the mechanism of complement action. I. Inhibition of complement activity by diisopropyl fluorophosphate. *J. Immunol.* 77:462, 1956.
- Berglund K, Laurell A-B, Nived O et al.: Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relations and changes on suppression of inflammation. *J. Clin. Lab. Immunol.* 4:7, 1980.
- Bischof P: Pregnancy-associated plasma protein-A: an inhibitor of the complement system. *Placenta* 2:29, 1981.
- Bokisch VA, Müller-Eberhard HJ, Cochrane CG: Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* 129:1109, 1969.
- Bokisch VA, Müller-Eberhard HJ: Anaphylatoxin inactivator of human plasma: its isolation and characterisation as a carboxypeptidase. *J. Clin. Invest.* 49:2427, 1970.

Bordet J: Sur l'agglutination et la dissolution des globules rouge par le sérum d'animaux injectés de sang défibriné. Ann. Inst. Pasteur (Paris) 12:688, 1898.

Borsos T: Immunoglobulin classes and complement-fixing activity. P 841 in Progress in Immunology. Ed. B Amos. Academic Press Inc., New York 1971.

Boyle MDP, Borsos T: The terminal stages of immune hemolysis - a brief review. Mol. Immunol. 17:425, 1980.

Brade V, Nicholson D, Bitter-Suermann D et al.: Formation of the C3-cleaving properdin enzyme on zymosan. Demonstration that factor D is replaceable by proteolytic enzymes. J. Immunol. 113:1735, 1974.

Brandslund I, Siersted HC, Svehaug S-E et al.: Double-decker rocket immunoelectrophoresis for direct quantitation of complement split products with C3d specificities in plasma. J. Immunol. Methods 44:63, 1981.

Budzko DB, Müller-Eberhard HJ: Cleavage of the fourth component of human complement (C4) by C1 esterase: Isolation and characterization of the low molecular weight product. Immunochemistry 7:227, 1970.

Chenoweth DE, Hugli TE: Demonstration of specific C5a receptor on intact human polymorphonuclear leucocytes. Proc. Nat. Acad. Sci. (Wash.) 75:3943, 1978.

Colten HR: Biosynthesis of complement. Adv. Immunol. 22:67, 1976.

Cooper NR: Immune adherence by the fourth component of complement. Science 165:396, 1969.

Cooper NR: Isolation and analysis of the mechanism of action of an inactivator of C4b in normal human serum. J. Exp. Med. 141:890, 1975.

Cooper NR, Jensen FC, Welsh RM et al.: Lysis of RNA tumor viruses by human serum: direct antibody-independent triggering of the classical complement pathway. J. Exp. Med. 144:970, 1976.

Cooper NR: Activation and regulation of the first complement component. Fed. Proc. 42:134, 1983.

Davis III AE, Zalut C, Rosen FS et al.: Human factor D of the alternative complement pathway. Physicochemical characteristics and N-terminal amino acid sequence. Biochemistry 18:5082, 1979.

DiScipio RG: The binding of complement proteins C5, factor B, $\beta 1H$, and properdin to C3b on zymosan. Biochem. J. 199:485, 1981.

DiScipio RG: The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology 45:578, 1982.

Donaldson VH, Hess EV, McAdams AJ: Lupus-erythematosus-like disease in three unrelated women with hereditary angioneurotic edema. Ann. Int. Med. 86:312, 1977a.

Donaldson VH, Rosen FS, Bing DH: Role of the second component of complement (C2) and plasma in kinin release in hereditary angioneurotic oedema (HANE) plasma. *Trans. Am. Assoc. Phys.* 40:174, 1977b.

Ellerson JR, Yasmeen D, Painter RH et al.: A fragment corresponding to the CH2 region of immunoglobulin G (IgG) with complement fixing activity. *FEBS Lett.* 24:318, 1972.

Esser AF: Interactions between complement proteins and biological and model membranes. P 277 in Biological membranes. Vol 4. Ed. D Chapman. Academic Press, 1981.

Fearon DT, Austen KF, Ruddy S: Formation of a hemolytically active cellular intermediate by the interaction between properdin factors B and D and the activated third component of complement. *J. Exp. Med.* 138:1305, 1973.

Fearon DT, Austen KF: Initiation of C3 cleavage in the alternative complement pathway. *J. Immunol.* 115:1357, 1975a.

Fearon DT, Austen KF: Properdin: binding to C3b and stabilization of the C3b dependant C3 convertase. *J. Exp. Med.* 142:856, 1975b.

Fearon DT, Daha MR, Weiler JM et al.: The natural modulation of the amplification phase of complement activation. *Transplant. Rev.* 32:12, 1976.

Fearon DT: Identification of the membrane glycoprotein that is the C3b receptor of the human, polymorphonuclear leucocyte, B lymphocyte and monocyte. *J. Exp. Med.* 152:10, 1980.

Fearon DT, Austen KF: The alternative pathway of complement: a system for host defense to microbial infection. *N. Engl. J. Med.* 303:259, 1980.

Fearon DT, Wong WW: Complement ligand-receptor interactions that mediate biological responses. *Ann. Rev. Immunol.* 1:243, 1983.

Ferrata A: Die Unwirksamkeit der komplexen Hämolyse in salzfreien Lösungen und ihre Ursache. Berlin. *Klin. Wschr.* 44:366, 1907.

Fey G, Colton HR: Biosynthesis of complement components. *Fed. Proc.* 40:2099, 1981.

Fodor J von: Die Fähigkeit des Blutes, Bakterien zu vernichten. *Dt. Med. Wschr.* 13:745, 1887.

Fong JSC, Renaud L: A hemolytic plate method for alternative pathway complement activity assay. *Amer. J. Clin. Path.* 69:156, 1978.

Forbes CD, Pensky J, Ratnoff OD: Inhibition of activated Hageman factor and activated plasma thromboplastin antecedant by purified serum C1 inactivator. *J. Lab. Clin. Med.* 76:809, 1970.

Forsgren A, McLean RH, Michael AF et al.: Studies on the alternative pathway in chelated serum. *J. Lab. Clin. Med.* 85:904, 1975.

Fujita T, Gigli I, Nussenzweig V: Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator. *J. Exp. Med.* 148:1044, 1978.

Gabriel DA, Hadler NM: The physical characterization of an aggregating IgG heteropolymer containing rheumatoid factor. *J. Biol. Chem.* 256:3240, 1981.

Gelfand JA, Boss CR, Conley CL et al.: Acquired C1 esterase inhibitor deficiency and angioedema. A review. *Medicine* 58:321, 1979.

Gewurz AT, Lint TF, Imherr SM et al.: Detection and analysis of inborn and acquired complement abnormalities. *Clin. Immunol. Immunopathol.* 23:297, 1982.

Gewurz H, Lint TF: Alternative modes and pathways of complement activation. P 27 in Comprehensive Immunology 2. Biological Amplification Systems in Immunology. Eds. NK Day, RA Good. Plenum Publishing Corporation, New York 1977.

Gigli I, Ruddy S, Austen KF: The stoichiometric measurement of the serum inhibitor of the first component of complement by the inhibition of immune hemolysis. *J. Immunol.* 100:1154, 1968.

Gigli I, Austen KF: Fluid phase destruction of C2^{hu} by C1^{hu}. I. Its enhancement and inhibition by homologous and heterologous C4. *J. Exp. Med.* 129:679, 1969.

Gigli I, Porter RR, Sim RB: The unactivated form of the first component of human complement, C1. *Biochem. J.* 157:541, 1976.

Gigli I, Fujita T, Nussenzweig V: Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc. Nat. Acad. Sci. (Wash.)* 76:6596, 1979.

Glass DN, Fearon DT, Austen KF: Inherited abnormalities of the complement system. P 1934 in The metabolic basis of inherited disease. Eds. JB Stanburg, JB Wyngaarden, DS Frederickson et al. McGraw-Hill, New York 1983.

Gorski JP: Quantitation of human complement fragment C4a in physiological fluids by a competitive inhibition radioimmune assay. *J. Immunol. Methods* 47:61, 1981.

Götze O, Müller-Eberhard HJ: The C3-activator system: an alternate pathway of complement activation. *J. Exp. Med.* 134:90s, 1971.

Götze O, Müller-Eberhard HJ: Alternative pathway of complement activation. *Adv. Immunol.* 24:1, 1976.

Hack CE, Hannema AJ, Eerenberg-Behmer AJ et al.: A C1 inhibitor complex assay (INCA): A method to detect C1 activation in vitro and in vivo. *J. Immunol.* 127:1450, 1981.

Hadler NM, Spitznagel JK, Quinet RJ: Lysosomal enzymes in inflammatory synovial effusions. *J. Immunol.* 123:572, 1979.

Halla JT, Volanakis JE, Schrohenloher RE: Immune complexes in rheumatoid arthritis sera and synovial fluids. A comparison of three methods. *Arthritis Rheum.* 22:440, 1979.

Harpel PC, Cooper NR: Studies on human plasma C $\bar{1}$ inactivator-enzyme interactions. *J. Clin. Invest.* 55:593, 1975.

Harpel PC, Cooper NR: Circulating C $\bar{1}$ inactivator-C $\bar{1}$ s-C $\bar{1}$ r complexes. Quantification by an enzyme-linked differential antibody immunosorbent assay. *Clin. Res.* 30:556A, 1982.

Hauptmann G: Genetic polymorphism of human complement proteins. *Blood Transfusion and Immunopathology*, Tome XXII, No 5, 1979.

Hedberg H: Studies on the depressed hemolytic complement activity of synovial fluid in adult rheumatoid arthritis. *Acta Rheum. Scand.* 9:165, 1963.

Hellsing K: The effects of different polymers for enhancement of antigen-antibody reaction as measured with nephelometry. *Protides Biol. Fluids* 21:579, 1973.

Hughes GRV: *Connective tissue diseases.* P 241. Blackwell Scientific Publications, Oxford 1977.

Hugli TE, Müller-Eberhard HJ: Anaphylatoxins: C3a and C5a. *Adv. Immunol.* 26:1, 1978.

Hugli TE, Chenoweth DE: Biologically active peptides of complement. Techniques and significance of C3a and C5a measurements. P 443 in Immunoassays: clinical techniques for the 1980s. Eds. RM Nakamura, WR Dito, ES Tucker. Alan R. Liss Inc., New York, 1980.

Humphrey JH, Dourmashkin RR: The lesions in cell membranes caused by complement. *Adv. Immunol.* 11:75, 1969.

Huntley CC, Robbins, JB, Lyerly AD et al.: Characterization of precipitating antibodies to ruminant serum and milk proteins in humans with selective IgA deficiency. *New Engl. J. Med.* 284:7, 1971.

Hurst MM, Volanakis JE, Hebster RB et al.: The structural basis for binding of complement by immunoglobulin M. *J. Exp. Med.* 140:1117, 1974.

Insenman DE, Podack ER, Cooper NR: The interaction of C5 with C3b in free solution: a sufficient condition for cleavage by a fluid phase C3/C5 convertase. *J. Immunol.* 124:326, 1980.

Jacob HS: The role of activated complement and granulocytes in shock states and myocardial infarction. *J. Lab. Clin. Med.* 98:645, 1981.

Johnson BA, Hoffmann LG: Effect of reduction and alkylation of rabbit IgG antibody. I. Effect on ability to activate complement depends on conditions of reduction. *Mol. Immunol.* 18:181, 1981.

Johnson PM, Watkins J, Holborow EJ: Antiglobulin production to altered IgG in rheumatoid arthritis. *Lancet* i:611, 1975.

Johnson PM, Faulk WP: Rheumatoid factor: its nature, specificity and production in rheumatoid arthritis. *Clin. Immunol. Immunopathol.* 6:414, 1976.

Johnson PM, Reading CA, Holborow EJ et al.: False-positive Rose-Waaler rheumatoid factor titre given by anti-ruminant IgM antibody. *Vox Sang.* 31:451, 1976.

Johnson U: Influence of ageing and polyethylene glycol on the quantitation of C3d/dg in plasma and serum. *Acta Path. Microbiol. Immunol. Scand. Sect. C*, in press, 1984.

Johnston Jr RB, Altenburger KM, Atkinson AW et al.: Complement in the newborn infant. *Pediatrics* 64:781, 1979.

Jones VE, Cowley PJ, Allen C et al.: The isolation of immune complexes containing IgM rheumatoid factor and recovery of IgG rheumatoid factor from the complexes. *J. Immunol. Methods* 37:1, 1980.

Kaplan RA, Curd JG, Deheer DH et al.: Metabolism of C4 and factor B in rheumatoid arthritis: Relation to rheumatoid factor. *Arthritis Rheum.* 23:911, 1980.

Kazatchkine MD, Fearon DT, Austen KF: Human alternative complement pathway: membrane associated sialic acid regulates the competition between B and beta-1-H for cell bound C3b. *J. Immunol.* 122:75, 1979.

Kazatchkine MD, Nydegger UE: The human alternative complement pathway: Biology and immunopathology of activation and regulation. *Prog. Allergy* 30:193, 1982.

Kerr MA: Limited proteolysis of complement components C2 and factor B. Structural analogy and limited sequence homology. *Biochem. J.* 183:615, 1979.

Kjaervig Broe M, Ingild A: Amplification of immunoprecipitates in agarose gels by horseradish peroxidase-labelled antibody. P 255 in Handbook of Immunoprecipitation-in-gel techniques. Ed. NH Axelsen. Blackwell Scientific Publications, Oxford 1983.

Kolb WP, Müller-Eberhard HJ: Mode of action of human C9: Adsorption of multiple C9 molecules to cell-bound C8. *J. Immunol.* 113:479, 1974.

Konno T, Katsuno Y, Hirai H: Change of electrophoretic mobility of purified alternative pathway factor D. *J. Immunol. Methods* 21:325, 1978.

Kunkel HG, Müller-Eberhard HJ, Fudenberg HH et al.: Gamma globulin complexes in rheumatoid arthritis and certain other conditions. *J. Clin. Invest.* 40:117, 1961.

Lachmann PJ, Thompson RA: Reactive lysis: The complement-mediated lysis of unsensitised cells. II. The characterisation of activated reactor as C5⁶ and the participation of C8 and C9. *J. Exp. Med.* 131:643, 1970.

Lachmann PJ, Hobart MJ: Complement technology. P 5A.1 in Handbook of Experimental Immunology. Ed. DM Weir. Blackwell Scientific Publications, Oxford, 1978.

Lachmann PJ, Peters DK: Complement. P 18 in Clinical aspects of Immunology. Vol. 1. Eds. PJ Lachmann, DK Peters. Blackwell Scientific Publications, Oxford 1982.

Lambert PH, Nydegger UE, Perrin LH et al.: Complement activation in seropositive and seronegative rheumatoid arthritis. ^{125}I -C1q binding capacity and complement breakdown products in serum and synovial fluid. *Rheumatology* 6:52, 1975.

Laurell A-B, Mårtensson U, Sjöholm AG: The development of simple tests for C1q, C1r, C1s, C2 and properdin. P 12 in Clinical aspects of the complement system. Eds. W Opferkuch, K Rother, DR Schultz. Georg Thieme Publishers, Stuttgart 1978.

Laurell A-B, Mårtensson U, Sjöholm AG: Quantitation of C1r-C1s-C1 inactivator complexes by electroimmunoassay. *Acta Path. Microbiol. Scand. Sect. C* 87:79, 1979.

Laurell C-B: Electroimmunoassay. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124:21, 1972.

Law SK, Levine RP: Interaction between the third complement protein and cell surface macromolecules. *Proc. Nat. Acad. Sci. (USA)* 74:2701, 1977.

Law SK, Lichtenberg NA, Holcombe FH et al.: Interaction between the labile binding sites of the fourth (C4) and fifth (C5) human complement proteins and erythrocyte cell membranes. *J. Immunol.* 125:634, 1980a.

Law SK; Lichtenberg NA, Levine RP: Covalent binding and hemolytic activity of complement proteins. *Proc. Nat. Acad. Sci. (USA)* 77:7194, 1980b.

Leikola J, Vyas GN: Human antibodies to ruminant IgM concealing the absence of IgA in man. *J. Lab. Clin. Med.* 77:629, 1971.

Lepow IH, Ratnoff OD, Rosen FS et al.: Observations on a proesterase associated with partially purified first component of human complement. *Proc. Soc. Exp. Biol. (N.Y.)* 92:32, 1956.

Lepow IH, Naff GB, Todd EW et al.: Chromatographic resolution of the first component of complement into three activities. *J. Exp. Med.* 117:983, 1963.

Lesavre PH, Müller-Eberhard HJ: Mechanism of action of factor D of the alternative complement pathway. *J. Exp. Med.* 148:1498, 1978.

Lesavre PH, Hugli TH, Esser AF et al.: The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* 123:529, 1979.

Levo Y, Fischel R, Ehrenfeld M: Circulating immune complexes in patients with rheumatoid arthritis: correlation with disease activity. *J. Rheumatol.* 8:851, 1981.

Loos M, Laurell A-B, Sjöholm AG et al.: Immunochemical and functional analyses of a complete C1q deficiency in man: Evidence that C1r and C1s are in the native form, and that they reassociate with purified C1q to form macromolecular C1. *J. Immunol.* 124:59, 1980.

Loos M: The classical complement pathway: Mechanism of activation of the first component by antigen-antibody complexes. *Prog. Allergy* 30:135, 1982.

Löfberg H, Grubb AO: Quantitation of γ -trace in human biological fluids; indications for production in the central nervous system. *Scand. J. Clin. Lab. Invest.* 39:619, 1979.

Löfberg H, Grubb AO, Sveger T et al.: The cerebrospinal fluid and plasma concentrations of γ -trace and β_2 -microglobulin at various ages and in neurological disorders. *J. Neurol.* 223:159, 1980.

Manni JA, Müller-Eberhard HJ: The eight component of human complement (C8): Isolation, characterization, and hemolytic efficiency. *J. Exp. Med.* 130:1145, 1969.

Manuel Y, Colle A, Leclercq M et al.: Low molecular weight proteinuria. *Contrib. Nephrol.* 1:156, 1975.

Martin A, Perassi R, Vottero E: Studies of hemolysis in an agar medium. *Publ. Health Lab.* 20:34, 1962.

Martin A, Lachmann PJ, Halbwachs L et al.: Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. *Immunochemistry* 13:317, 1976.

Mayer MM: Development of the one-hit theory of immune hemolysis. P 268 in Immunochemical approaches to problems in microbiology. Eds. M Heidelberger, OJ Plesica, Rutgers University Press, New Brunswick N.J. 1961.

Mayer MM: Complement and complement fixation. P 133 in Experimental immunochemistry. Ed. EA Kabat. Charles C. Thomas Publisher, Springfield Ill. 1971.

Mayes JT, Schreiber RD, Cooper NJ: Development and application of an enzyme linked immunosorbent assay for quantitation of alternative complement pathway activation in human serum. *Clin. Res.* 31:539 A, 1983.

McCabe WR: Serum complement levels in bacteremia due to gram negative organisms. *New Engl. J. Med.* 288:21, 1973.

McDougal JS, Hubbard M, McDuffie FC et al.: Comparison of five assays for immune complexes in rheumatic diseases. An assessment of their validity for rheumatoid arthritis. *Arthritis Rheum.* 25:1156, 1982.

Merino J, Rodriguez-Valverde V, Lamelas JA: Prevalence of deficits of complement components in patients with recurrent meningococcal infections. *J. Inf. Dis.* 148:331, 1983.

Milgrom F, Millers R: Serological hemolysis and complement fixation test in agar gel. *Vox Sang.* 8:537, 1963.

Milgrom H, Curd JG, Kaplan RA et al.: Activation of the fourth component of complement (C4): Assessment by rocket immunoelectrophoresis in correlation with metabolism of C4. *J. Immunol.* 124:2780, 1980.

Morris KM, Colton HR, Bing DH: The first component of complement. A quantitative comparison of its biosynthesis in culture by human epithelial and mesenchymal cells. *J. Exp. Med.* 148: 1007, 1978.

Müller-Eberhard HJ, Calcott MA: Interaction between C1q and γ G globulin. *Immunochemistry* 3:500, 1966.

Müller-Eberhard HJ, Polley MJ, Calcott MA: Formation and functional significance of molecular complex derived from the second and fourth component of human complement. *J. Exp. Med.* 125:359, 1967.

Müller-Eberhard HJ: Chemistry and reaction mechanisms of complement. *Adv. Immunol.* 8:1, 1968.

Müller-Eberhard HJ: Complement. *Ann. Rev. Biochem.* 38:389, 1969.

Müller-Eberhard HJ, Schreiber RD: Molecular biology and chemistry of the alternative pathway of complement. *Adv. Immunol.* 29:1, 1980.

Naff GB, Pensky J, Lepow IH: The macromolecular nature of the first component of human complement. *J. Exp. Med.* 119:593, 1964.

Nilsson UR, Mandle RJ, McConnell-Mapes JA: Human C3 and C5: subunit structure and modifications by trypsin and C42 - C423. *J. Immunol.* 114:815, 1975.

Nuttall G: Experimente über die bakterien-feindlichen Einflüsse des tierischen Körpers. *Z. Hyg. Infect.-Kr.* 4:353, 1888.

Nydegger UE, Zubler RA, Gabay R et al.: Circulating complement breakdown products in patients with rheumatoid arthritis. Correlation between plasma C3d, circulating immune complexes, and clinical activity. *J. Clin. Invest.* 59:862, 1977.

Nydegger UE, Fearon DT, Austen KF: The modulation of the alternative pathway of complement in C2-deficient human serum by changes in concentration of the component and control proteins. *J. Immunol.* 120:1404, 1978.

Opferkuch W, Berger V, Kapp S et al.: Standard test values and reproducibility of complement determinations. P 4 in Clinical aspects of the complement system. Eds. W. Opferkuch, K. Rother, DR Schultz, Georg Thieme Publishers, Stuttgart, 1978.

Pangburn MK, Schreiber RD, Müller-Eberhard HJ: Human complement C3b inactivator: Isolation, characterization, and demonstration of an absolute requirement for the serum protein β 1H for cleavage of C3b and C4b in solution. *J. Exp. Med.* 146:257, 1977.

Pangburn MK, Müller-Eberhard HJ: Complement C3 convertase: cell surface restriction of beta-1-H control and generation of restriction on neuramidase treated cells. *Proc. Nat. Acad. Sci. (USA)*. 75:2416, 1978.

Pangburn MK, Müller-Eberhard HJ: Relation of a putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. *J. Exp. Med.* 152:1102, 1980.

Patrick RA, Taubman SB, Lepow IH: Cleavage of the fourth component of human complement (C4) by activated C1s. *Immunochemistry* 7:217, 1970.

Pekin TJ, Zvaifler NJ: Hemolytic complement in synovial fluid. *J. Clin. Invest.* 43:1372, 1964.

Perrin LH, Nydegger UE, Zubler RH et al.: Correlation between levels of breakdown products of C3, C4, and properdin factor B in synovial fluids from patients with rheumatoid arthritis. *Arthritis Rheum.* 20:647, 1977.

Pfeiffer R, Issaef R: Über die spezifische Bedeutung der Choleraimmunität. *Z. Hyg. Infekt.-Kr.* 17:355, 1894.

Pillemer L, Blum L, Lepow IH et al.: The properdin system and immunity: I. Demonstration and isolation of a new serum protein and its role in immune phenomena. *Science* 120:279, 1954.

Pisko EJ, Turner RA, Foster SL: Induction of human rheumatoid factor producing cells by aggregated IgG. *Arthritis Rheum.* 25:1108, 1982.

Podack ER, Kolb WP, Müller-Eberhard HJ: The C5b-6 complex: formation, isolation, and inhibition of its activity by lipoprotein and the S-protein of human serum. *J. Immunol.* 120:1841, 1978.

Podack ER, Kolb WP, Esser AF et al.: Structural similarities between C6 and C7 of human complement. *J. Immunol.* 123:1071, 1979.

Polhill RB Jr, Newman SL, Pruitt KM et al.: Kinetic assessment of alternative pathway activity in a hemolytic system. II. Influence of antibody on alternative pathway activation. *J. Immunol.* 121:371, 1978.

Polley MJ, Müller-Eberhard HJ: The second component of human complement: its isolation, fragmentation by C'1 esterase, and incorporation into C'3 convertase. *J. Exp. Med.* 128:533, 1968.

Pope RM, Teller DC, Mannik M: The molecular basis of selfassociation of IgG-rheumatoid factors. *J. Immunol.* 115:365, 1975.

Porter RR: Structure and activation of early components of complement. *Fed. Proc.* 36:2191, 1977.

Rapp HJ, Borsos T: Molecular basis of complement action. Appleton-Century-Crofts, New York 1970.

Ratnoff OD, Pensky J, Ogston D et al.: The inhibition of plasma kallikrein, plasma permeability factor and the C1r subcomponent of the first component of complement by serum C'1 esterase inhibitor. *J. Exp. Med.* 129:315, 1969.

Reid KBM, Lowe DM, Porter RR: Isolation and characterization of C1q, a subcomponent of the first component of complement from human and rabbit sera. *Biochem. J.* 130:749, 1972.

Reid KBM, Porter RR: The proteolytic activation systems of complement. *Ann. Rev. Biochem.* 50:433, 1981.

Romeyn JA, Baragar FD, Cook J: Anti-immunoglobulin analysis by diffusion patterns of inhibition and facilitation of complementary lysis in agar. III. Anti-immunoglobulin diffusion-lysis patterns of normal human and rheumatoid sera. *J. Immunol. Methods* 7:47, 1975.

Rose HM, Ragan C, Pearce E et al.: Differential agglutination of normal and sensitized erythrocytes by sera of patients with rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med.* 68:1, 1948.

Ross GD: Structure and function of membrane complement receptors. *Fed. Proc.* 41:3089, 1982.

Ross GD, Lambris JD, Cain JA et al.: Generation of three different fragments of bound C3 with purified factor I or serum. I. Requirements for factor H vs CR1 cofactor activity. *J. Immunol.* 129:2051, 1982.

Ruddy S, Austen KF: C3b inactivator of man. II. Fragments produced by C3b inactivator cleavage of cell-bound or fluid phase C3b. *J. Immunol.* 107:742, 1971.

Ruddy S, Carpenter CB, Chin KW et al.: Human complement metabolism: an analysis of 144 studies. *Medicine* 54:165, 1975.

Sabharwal UK, Vaughan JH, Fong S et al.: Activation of the classical pathway of complement by rheumatoid factors: assessment by radioimmunoassay for C4. *Arthritis Rheum.* 25:161, 1982.

Scharfstein J, Ferreira A, Gigli I et al.: Human C4-binding protein. I. Isolation and characterization. *J. Exp. Med.* 148:207, 1978.

Schenkein HA, Ruddy S: The role of immunoglobulins and alternative pathway activation by zymosan. II. The effect of IgG on the kinetics of the alternative pathway. *J. Immunol.* 126:11, 1980.

Schreiber RD, Müller-Eberhard HJ: Fourth component of human complement: description of a three polypeptide chain structure. *J. Exp. Med.* 140:1324, 1974.

Schur PH, Austen KF: Complement in human disease. *Ann. Rev. Med.* 19:1, 1968.

Schutte M, DiCamelli R, Murphy P et al.: Effects of anesthesia, surgery and inflammation upon host defense mechanisms. I. Effects upon the complement system. *Int. Archs. Allergy Appl. Immun.* 48:706, 1975.

Shiraishi S, Stroud RM: Cleavage products of C4b produced by enzymes in human serum. *Immunochemistry* 12:935, 1975.

Sjöholm AG: Complement components in normal serum and plasma quantitated by electroimmunoassay. *Scand. J. Immunol.* 4:25, 1975.

Sjöholm AG, Braconier JH, Söderström C: Properdin deficiency in a family with fulminant meningococcal infections. *Clin. Exp. Immunol.* 50:291, 1982.

Soothill JF, Harvey BAM: A defect of the alternative pathway of complement. *Clin. Exp. Immunol.* 27:30, 1977.

Strunk RC, Fenton LJ, Gaines JA: Alternative pathway of complement activation in full term and premature infants. *Pediatr. Res.* 13:641, 1979.

Sturfelt G, Sjöholm AG: Cleavage of C2 in pathological serum and plasma studied by crossed immunoelectrophoresis. *Immunobiol.* 164:302, 1983.

Sturfelt G, Truedsson L, Thysell H et al.: Serum level of complement factor D in systemic lupus erythematosus (SLE) - an indicator of glomerular filtration rate. *Acta Med. Scand.* in press, 1984.

Sundomo JS: The leukocyte complement system. *Fed. Proc.* 41:3094, 1982.

Takahashi M, Takahashi S, Hirose S: Solubilization of antigen-antibody complexes: a new function of complement as a regulator of immune reactions. *Prog. Allergy* 27:134, 1980.

Tanimoto K, Cooper NR, Johnson JS et al.: Complement fixation by rheumatoid factor. *J. Clin. Invest.* 55:437, 1975.

Tarkowski A, Bjursten LM, Nilsson L-Å et al.: False positive results in class-specific rheumatoid factor (RF) assays due to interaction between RF and Fc fragments of anti-immunoglobulin indicator reagents. *J. Immunol. Methods* 58:171, 1983.

Thompson RA, Rowe DS: Immune haemolysis in agar: demonstration of the protective action of antibodies. *Immunology* 13:411, 1967.

Thompson RA, Lachmann PJ: Reactive lysis: the complement-mediated lysis of unsensitized cells. I. The characterisation of the indicator factor and its identification as C7. *J. Exp. Med.* 131:629, 1970.

Tomasi TB, Katz L: Human antibodies against bovine immunoglobulin M in IgA deficient sera. *Clin. Exp. Immunol.* 9:3, 1971.

Trentham DE: Collagen arthritis as a relevant model for rheumatoid arthritis: evidence pro and con. *Arthritis Rheum.* 25:911, 1982.

Truedsson L, Sturfelt G: False-positive Waaler-Rose test due to anti-rabbit IgM antibodies in IgA deficiency. *Clin. Exp. Rheum.* 1:307, 1983.

Ueno S: Studien über die Komponenten des Komplementes. I. Über die Bindung des Mittelstücks bei der Hämolyse. *Jap. J. Med. Sci., VII. Social Med. Hyg.* 2:201, 1938a.

Ueno S: Studien über die Komponenten des Komplementes. II. Über die Bindung der 4 Komponente bei der Hämolyse. *Jap. J. Med. Sci., VII. Social Med. Hyg.* 2:225, 1938b.

Vaughan JH, Bayles TB, Favour CB: Serum complement in rheumatoid arthritis. *Am. J. Med. Sci.* 222:186, 1951.

Versey JM, Hobbs JR, Holt PJL: Complement metabolism in rheumatoid arthritis. *Ann. Rheum. Dis.* 32:557, 1973.

Vogt W, Dames W, Schmidt G et al.: Complement activation by the properdin system: formation of a stoichiometric C3 cleaving complex of properdin factor B with C3b. *Immunochemistry* 14:201, 1977.

Vogt NG, Schmidt B, Buttlar HB van et al.: A new function of the activated third component of complement: binding to C5, an essential step for C5 activation. *Immunology* 34:29, 1978.

Volanakis JE, Schrohenloher RE, Stroud RM: Human factor D of the alternative complement pathway: purification and characterization. *J. Immunol.* 119:337, 1977.

Waalder E: On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. *Acta Path. Microbiol. Scand.* 17:172, 1940.

Weiler JM, Daha MR, Austen KF et al.: Control of the amplification convertase of complement by the plasma protein β 1H. *Proc. Nat. Acad. Sci. (Wash.)* 73:3268, 1976.

Weinstein A, Peters K, Brown D et al.: Metabolism of the third component of complement (C3) in patients with rheumatoid arthritis. *Arthritis Rheum.* 15:49, 1972.

Weinstein MP, Gocke DJ, Gewurz A: Complement deficiency and sporadic meningococcal disease. *New Engl. J. Med.* 309:615, 1983.

Wells JV, Bleumers JF, Fudenberg HH: Human anti-IgM iso-antibodies in subjects with selective IgA deficiency. *Clin. Exp. Immunol.* 12:305, 1972.

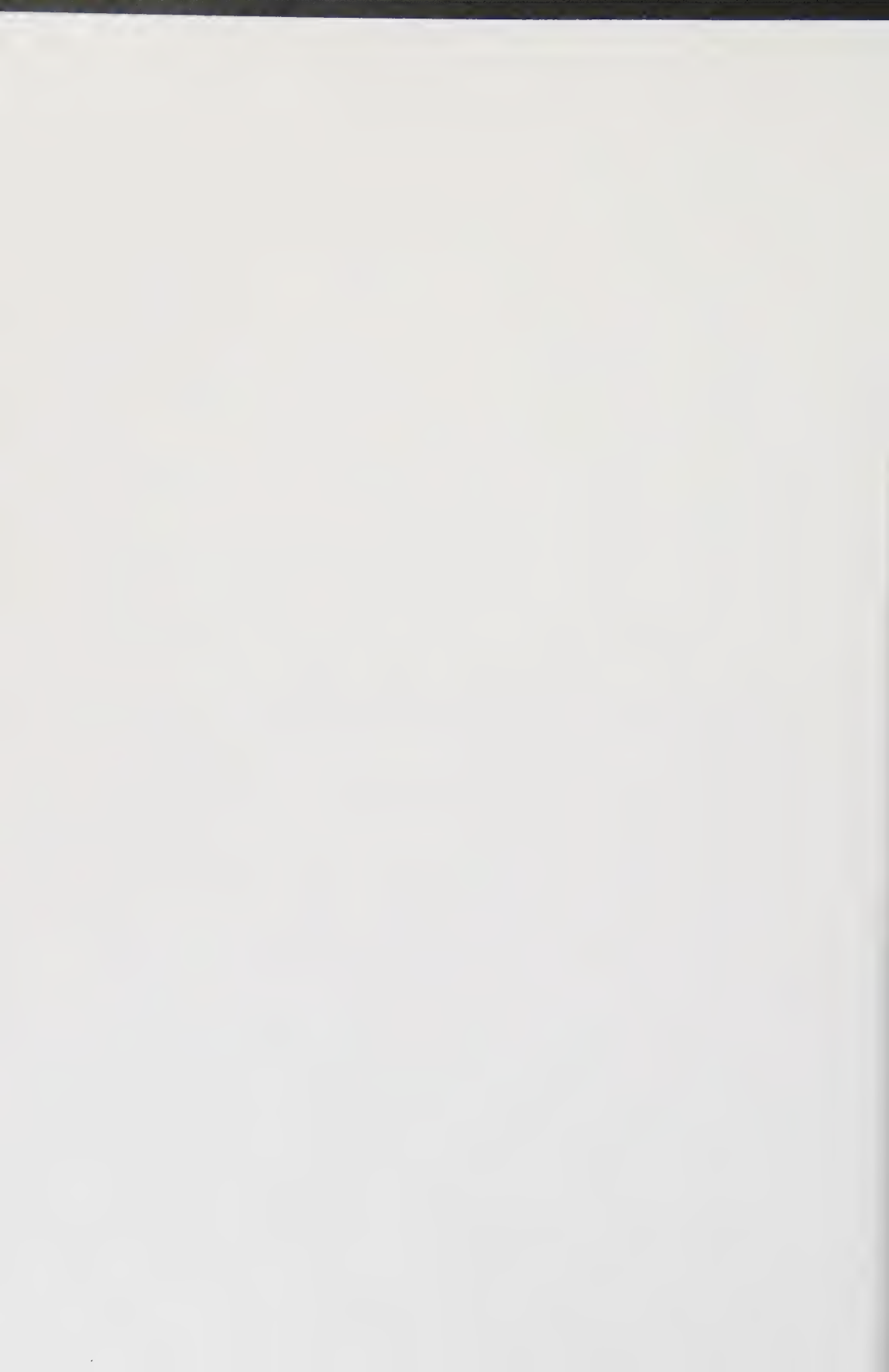
Whaley K, Thompson RA: Requirement for β 1H globulin and C3b inactivator in the control of the alternative complement pathway in human serum. *Immunology* 35:1045, 1978.

Wilson WA, Thomas, EJ, Sissons JGP: Complement activation in asymptomatic patients with sickle cell anemia. *Clin. Exp. Immunol.* 36:130, 1979.

Yonemasu K, Stroud RM: Structural studies on human C1q: Non-covalent and covalent subunits. *Immunochemistry* 9:545, 1972.

Ziccardi RJ, Cooper NR: Activation of C1r by proteolytic cleavage. *J. Immunol.* 116:504, 1976.

A P P E N D I X (I - VII)



SCREENING FOR DEFICIENCIES IN THE CLASSICAL AND ALTERNATIVE PATHWAYS OF COMPLEMENT BY HEMOLYSIS IN GEL

L. TRUEDSSON, A. G. SJÖHOLM and A.-B. LAURELL

Institute of Medical Microbiology, University of Lund, Sweden

Truedsson, L., Sjöholm, A. G. & Laurell, A.-B. Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. *Acta path. microbiol. scand. Sect. C*, 89: 161-166, 1981.

Two assays based on hemolysis in gel were assessed for screening complement (C) component deficiencies. In one assay sensitized sheep erythrocytes (EA) were incorporated in agarose gel containing Ca^{2+} and Mg^{2+} , in the other guinea pig erythrocytes (GpE) were used in the presence of Mg^{2+} and EGTA. With few exceptions, fresh samples from healthy individuals produced homogenous areas of complete hemolysis in both assays. Clearly aberrant patterns were observed in approximately 4% of healthy blood donors. Sera from patients having complete deficiencies of C1q, C2 or C4 produced clear lysis of GpE only, whereas in sera lacking C3 or C8 lysis was grossly impaired in both assays. Properdin deficient serum produced very slight lysis of GpE but normal lysis of EA. Reconstitution of these C-deficient sera gave normal lysis. Together, the two assays supplement immunochemical C3 and C4 determinations for screening out C disorders.

Key words: Hemolysis in gel; complement screening; complement deficiencies.

L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 4.xi.80 Accepted 27.xi.80

In clinical laboratory work, the function of the classical pathway of complement (C) is most often measured by immune hemolysis using the CH_{50} test (9, 17). For the alternative pathway, many assays have been proposed (3, 4, 6, 9, 15, 21). In general, the time and skill required for such assays make them unsuitable for large screening purposes.

Methods based on hemolysis in gel (HIG) have proved useful for quantitative estimation of several C components (9). HIG of sensitized sheep erythrocytes by the classical pathway and of rabbit or guinea pig erythrocytes by the alternative pathway has also been described for assessment of whole C (3, 5, 9). However, the efficiency of such screening tests in detecting disorders of C has not been clearly documented. In the present study, normal serum and plasma samples, sera with selective C component deficiencies and various pathological sera were examined using HIG with sensitized sheep erythro-

cytes and guinea pig erythrocytes for screening of the classical and alternative pathways, respectively.

MATERIALS AND METHODS

Buffers. Veronal-buffered saline with Mg^{2+} and Ca^{2+} (VBS++) or without cations (VBS) was prepared by standard methods (17).

EGTA (ethylene glycol bis-amino tetraacetic acid, Sigma Chemicals, St. Louis, Mo., USA) was dissolved at alkaline pH and adjusted to 0.2M, pH 7.4. This stock solution was diluted in VBS to a final concentration of 10 mM EGTA. Mg^{2+} was added in the stated concentrations. The buffer (VBS- Mg^{2+} -EGTA) was adjusted to pH 7.4 with 1M NaOH.

Sheep erythrocytes (E). The blood was first stored in Alsever's solution at 4 °C for one week and could then be used for about 10 weeks. E were sensitized with whole rabbit anti-sheep hemolysin (SBL, Stockholm, Sweden), which mainly contained IgM antibodies. The lowest dose of hemolysin that did not limit lysis of the

cells by guinea pig serum was used for optimal sensitization (17).

Guinea pig erythrocytes (GpE). The blood was handled in the same way as for sheep blood and could then be used for at least three weeks.

Performance of the assays. Sensitized sheep erythrocytes (EA) were suspended in 0.6% agarose (Agarose (ME), Marine Colloids Inc) in VBS++ at about 50 °C to a final concentration of 1.0%. The mixture was poured between two glass slides, 105 × 205 mm, and allowed to set. The space between the glass slides was 1.0 mm. After removal of one of the glass slides, 3.0 mm wells were cut in the gel and 5.0 µl samples were applied to each well. Incubation in moist atmosphere was carried out at 4 °C for 18 hours to allow diffusion to occur and then at 37 °C for 2 hours for the lytic reaction. The plates could be stored for at least 5 days at 4 °C before use. To preserve the lytic patterns obtained, a filter paper moistened with physiological NaCl plus a 1 cm thick wad of soft absorbant paper was placed over the gel and a weight (2.5–10 g/cm²) was placed on top to exert a gentle, even pressure over the entire gel surface, for 15 minutes. The filter paper was then carefully removed and the plates left to air-dry. The plates used could accommodate 60–80 samples.

The procedure for the GpE assay was the same however, VBS-Mg²⁺-EGTA was used as buffer. After diffusion at 4 °C for 18 hours, lytic zones were developed by incubation at 37 °C for 2–3 hours. The plates were kept at 4 °C during the application of samples. The plates were used the same day they were prepared to avoid disturbing background lysis.

Normal serum and plasma. Blood specimens were obtained from 105 registered blood donors, mainly males. The blood was collected in two tubes, one without anticoagulant, the other with Na₂EDTA in a final concentration of 4 mM. Centrifugation was carried out at room temperature. Samples were frozen at –80 °C within 5 hours of collection.

Sera with selective deficiencies of C components. Sera from three patients with complete C1q deficiency were examined. One patient has been described earlier (13). C1q deficient sera from two Yugoslavian siblings were referred to us by Dr. Mikuška, Belgrade. Sera from patients with homozygous deficiencies of C2, C4 (8), C3 (18) or C8 (2) were tested, as was also the serum from a young man with complete lack of properdin (1).

Deficiencies of control proteins. C $\bar{1}$ inactivator (C $\bar{1}$ IA) deficient serum from a patient with hereditary angioedema (HAE) and serum from a patient (24) with homozygous deficiency of C3b inactivator (C3b INA) were examined.

Other pathological sera. Sera from two patients with chronic urticaria were studied (11). Sera were also examined from three patients with hypocomplementemic glomerulonephritis. One was from a girl with partial lipodystrophy, and membranoproliferative glomerulonephritis showing the presence of C3 nephritic factor. The others were obtained from patients in the early phase of acute poststreptococcal glomerulonephritis. In addition sera from patients with rheumatoid arthritis,

myeloma, primary biliary cirrhosis, acute cholecystitis with cholestasis and Crohn's disease were selected and included in the study.

Immunochemical methods. C1q, C1r, C1s, C2, C4, C3, C8, properdin, C $\bar{1}$ IA (12, 20) and C3b INA were determined by electroimmunoassay. Crossed immunoelectrophoresis was used for detection of C1r–C1s complexes and C $\bar{1}$ r–C $\bar{1}$ s–C $\bar{1}$ IA complexes (10, 11).

Purified C components. C1q was prepared according to Yonemasu & Stroud (25). Functionally pure C2 and C4 were prepared as described previously (10). C2 was further purified on hydroxylapatite (16). C3 was prepared according to Tack & Prahl (22). C8 was prepared as described by Manni & Müller-Eberhard (14). Native properdin was prepared according to Götze *et al.* (7) with omission of the final immune absorption step.

RESULTS

Normal Serum and EDTA Plasma

Fresh samples from healthy individuals produced homogenous areas of complete lysis in both assays (Fig. 1 & 2). Differences between serum and plasma were negligible. In the assay using GpE and VBS-Mg²⁺-EGTA various concentrations of Mg²⁺ were tried. The appearance of the hemolytic areas produced by serum or plasma when Mg²⁺ was used at 2.5 mM to 10 mM was similar. In subsequent experiments Mg²⁺ was used at 5 mM. Unless the plates containing GpE were cooled during application of samples, the lytic areas produced by some samples were clearly diminished. This was not observed with plates containing EA.

Serum and plasma from 105 healthy blood donors were examined. Of these, three with normal lysis of EA had impaired lysis of GpE in that no lysis was seen in one case and partial lysis of the cells was produced in two cases. Serum and plasma from one donor showed a sharply outlined zone of unlysed cells in the assay with EA. This zone was surrounded by a small inner area and a peripheral ring of clear lysis. Samples from 96 donors produced complete lysis in both assays. In 5 donors slightly impaired lysis, with traces of unlysed cells near the application well, was observed with serum or plasma in one or both of the assays. Thus, using the two assays for screening of apparently healthy individuals, clearly aberrant patterns were found in approximately 4%.

Handling of Samples

Storage of normal serum at room temperature for two days did not alter the lytic patterns obtained in either assay. Normal plasma kept at room temperature for two days showed no difference in the lytic patterns observed with GpE. In some cases impaired hemolytic activity was noted in the assay

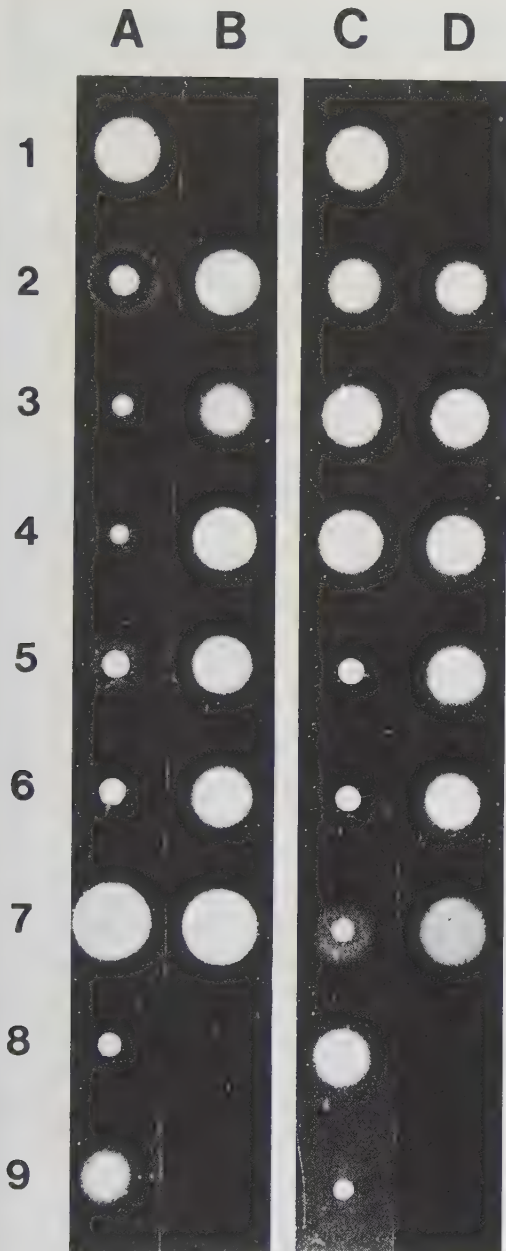


Fig. 1. Hemolysis in gel assays using EA (A&B) and GpE (C&D). Results with reconstituted sera are shown in B and D. 1. Normal human serum. 2. C1q deficient serum. 3. C2 deficient serum. 4. C4 deficient serum. 5. C3 deficient serum. 6. C8 deficient serum. 7. Properdin deficient serum. 8. HAE serum. 9. C3b INA deficient serum.

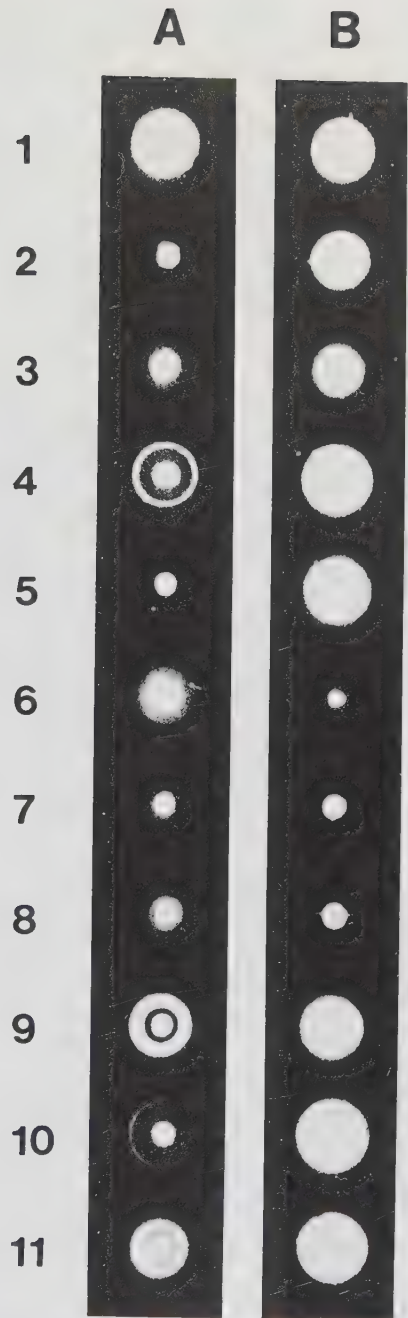


Fig. 2. Hemolysis in gel assays using EA (A) and GpE (B). 1. Normal human serum. 2. Serum with C1q less than 5% and high amounts of C1r-C1s complexes and 3. serum with normal C1q and all C1r and C1s present in C1r-C1s-CT IA complexes from two patients with chronic urticaria. 4-11. Pathological sera from patients with: 4. Rheumatoid arthritis. 5. Myeloma. 6 and 7. Acute glomerulonephritis. 8. Membranoproliferative nephritis. 9. Primary biliary cirrhosis. 10. Acute cholecystitis with cholestasis. 11. Crohn's disease.

using EA when plasma stored more than one day at room temperature was examined. Serum and plasma showed a decrease in hemolytic activity after storage for 5 days. This was more pronounced in plasma samples. Repeated freezing at -80°C and thawing up to 5 times before analysis was performed on aliquots of normal and various pathological serum and plasma samples. The samples were thawed by keeping them at room temperature for one hour. This treatment of the samples did not appreciably affect the results.

Sera with Selective Deficiencies of C Components, (Fig. 1)

Sera from patients with complete deficiencies of C1q, C2 and C4 produced complete lysis only of GpE. C1q deficient sera from the two Yugoslavian siblings gave a diffuse peripheral ring of partial lysis in the EA assay (Fig. 1). The third C1q deficient serum (13) produced no lysis in this assay. Serum lacking C3 demonstrated no lysis of GpE and the lysis of EA was hardly discernible. C8 deficient serum showed a complete lack of hemolysis in both assays. Properdin deficient serum induced a small area of partial lysis of GpE but normal lytic activity with EA. Prolonged incubation of the plate at 37°C for three hours or more, resulted in a slightly more pronounced lysis of GpE observed with the properdin deficient serum.

Deficiencies of Control Proteins, (Fig. 1)

Sera from patients with HAE gave lysis of GpE alone and C3b INA deficient serum produced a small area of partial lysis in the EA assay but no lysis of GpE.

Reconstitution experiments, (Fig. 1)

Sera from individuals with complete deficiencies of C1q, C2, C4, C3, C8 or properdin were

reconstituted with purified C components to approximate normal serum concentrations, after which normal lytic activity was obtained with all the sera except one of the C1q deficient sera (13).

Other pathological sera, (Fig. 2)

The levels of C1q, C1s, C4 and C3 in the sera are provided in Table 1. Two sera from patients with chronic urticaria were examined. One with C1q less than 5% and high amounts of C1r-C1s complexes produced no lysis of EA. The second serum containing normal C1q but with C1r and C1s present as C1r-C1s-C1 IA complexes produced a small faint lytic area with EA. Both these sera gave complete lysis of GpE. Sera from patients with rheumatoid arthritis, primary biliary cirrhosis, acute cholecystitis with cholestasis and Crohn's disease produced patterns of lysis, characterized by the appearance of non lysed zones in the EA assay but complete lysis of GpE. Lytic activity with EA was absent in serum from a patient with myeloma. This serum lysed GpE in a normal fashion. Sera from two patients with acute glomerulonephritis and serum from the patient with partial lipodystrophy and membrano-proliferative nephritis caused small areas of partial lysis with EA but no lysis of GpE.

DISCUSSION

The usefulness of simple screening tests for whole complement is obvious. In the present study, two methods based on hemolysis in gel (HIG) were tested to assess the functional activity of C in serum or EDTA plasma. These methods were capable of distinguishing between defects of classical and alternative pathway activation and of the terminal effector sequence, common to the two pathways. Thus, lysis of sensitized sheep erythrocytes (EA) but

TABLE 1. C1q, C1s, C4 and C3 in Sera Studied by HIG Assays (See Fig. 2). Values in Percent of Normal

	C1q	C1s	C4	C3
Chronic urticaria	<5	92	32	50
Chronic urticaria	122	96	38	75
Rheumatoid arthritis	104	106	130	120
Myeloma	<5	25	15	85
Acute glomerulonephritis	107	104	48	14
Acute glomerulonephritis	100	94	78	9
Membranoproliferative nephritis	82	92	72	7
Primary biliary cirrhosis	120	90	270	180
Acute cholecystitis with cholestasis	91	99	270	180
Crohns disease	102	120	95	105
Normal range (100 blood donors)	78-130	79-139	53-207	70-136

not of unsensitized guinea pig erythrocytes (GpE) was grossly impaired in sera deficient in C1q, C4, or C2, while properdin deficient serum gave the reversed pattern. The reason for the peripheral zone of partial lysis with two of the C1q deficient sera is not obvious. Serum deficient in C3 or C8 failed to produce lysis in both assays. Reconstitution of the deficient sera with the missing purified components provided normal patterns of lysis with exception of one C1q deficient serum. Hypocomplementemia secondary to C1 IA deficiency in HAE or C3b INA deficiency could also be demonstrated by these methods.

In the assay using EA the classical pathway is initiated by the reaction of C1 with cell bound antibody. In the alternative pathway assay the mechanism is less clear. GpE are not alternative pathway activators but are considered to be lysed through »bystander lysis« by late components in the C sequence, the activation being brought about by Mg^{++} in the fluid phase and by the agarose (9).

No essential differences were observed between the lytic patterns obtained with serum or EDTA plasma. Samples from 105 healthy blood donors gave homogenous areas of clear lysis in both assays, with few exceptions. One donor demonstrated a sharply outlined ring of unlysed EA cells within inner and outer zones of lysis. This was similar to the »target« pattern described by Thomson & Rowe (23), who in some sera found the pattern to be due to natural IgG anti-sheep antibodies. In the assay using GpE, three showed no or partial lysis. Functional defects of the alternative pathway have been reported to be more common than generally recognized (19, 21).

Sera from patients with chronic urticaria and previously demonstrated functional C1 disorder (11) and from glomerulonephritis patients with depletion of C3 and alternative pathway components, also showed impaired lysis of EA and of GpE, respectively. A few sera tested from patients with rheumatoid arthritis, primary biliary cirrhosis, acute cholecystitis with cholestasis or Crohn's disease gave lysis of EA with pronounced inner zones of unlysed cells. Serum from a patient with myeloma failed to lyse EA. These findings suggest that the screening procedure used might be informative not only for demonstration of profound hypocomplementemia but perhaps also in the detection of anticomplementary substances. Preliminary experiments indicate that anti-sheep cell antibodies were not responsible in every case. A more detailed study of sera producing inhibition of lysis is in progress.

The reproducibility of the HIG assays was high. With normal serum and plasma, samples could be

stored at room temperature for one or two days. Repeated freezing at -80°C a few times did not appreciably influence the results. In studies of pathological sera showing impaired lysis that may be due to anticomplementary substances, it might be advisable to confirm the findings with a new sample before an extended analysis is performed.

The size of the lytic areas in HIG is dose dependent, and the method has been tested for measuring whole C by immune hemolysis (5). A quantitative approach, in our opinion seems questionable, particularly when pathological sera are studied, as varying types of lytic patterns – partial lysis, appearance of nonlytic zones etc – may be compromising.

It should be emphasized that the screening assays described here do not consistently reveal moderate degrees of hypocomplementemia. The levels of C components such as C4 may be substantially decreased in sera that yield normal lytic patterns in the HIG assays.

In our laboratory, screening by HIG is used to supplement immunochemical C3 and C4 determinations in routine diagnostic work. If decreased levels of C3 and C4 – as an expression for C activation – do not provide a sufficient explanation for impaired lysis in the HIG tests, further analysis is carried out as follows:

1. Impaired lysis of EA, but normal lysis of GpE: immunochemical determination of C1q, C1r, C1s, C2, C1 IA and of C1r–C1s–C1 IA complexes.

2. Impaired lysis of GpE, but normal lysis of EA: measurement of factor B and properdin and assessment of alternative pathway activation by inulin treatment of serum (6).

3. Impaired lysis in both assays: immunochemical analysis of C5, C6, C7, C8, C9, C3b INA and $\beta 1\text{H}$. In some situations, additional functional tests are performed.

The screening method is quite simple and might well be performed together with immunochemical C3 and C4 determinations in laboratories not specialized in C studies. This could facilitate the selection of patients for extended analysis of C, which might help to widen our knowledge of C deficiencies and the relationships between C disorders and disease.

This work was supported by grants from the Swedish Medical Research Council (B81-16X-68), Magn. Bergvalls Stiftelse, Föreningen för bistånd åt Vanföra i Skåne, Greta och Johan Kocks Stiftelser, Konung Gustaf V:s 80-års fond and Riksförbundet mot reumatism.

REFERENCES

1. Braconier, J.-H., Sjöholm, A. G. & Söderström, C.: Meningococcal infections in a family: possible association with deficiency of properdin. Unpublished results.
2. Ellis-Pegler, R. B. et al.: Deficiency of the eight component of complement and recurrent meningococcal disease: A case and family study. Unpublished results.
3. Fong, J. S. C. & Renaud, L.: A hemolytic plate method for alternative pathway complement activity assay. *Amer. J. Clin. Path.* 69: 156–160, 1978.
4. Forsgren, A., McLean, R. H., Michael, A. F. & Ouie, P. G.: Studies of the alternate pathway in chelated serum. *J. Lab. clin. Med.* 85: 904–912, 1975.
5. Ghanta, V. K., McGhee, J. R., Soong, S.-J., Hamlin, N. M., Hurst, D. C. & Hiramoto, R. N.: A study of the single radial hemolysis in gel system – III. Quantitation of complement. *Immunochemistry* 10: 645–649, 1973.
6. Göze, O. & Müller-Eberhard, H. J.: The C3-activator system: an alternate pathway of complement activation. *J. exp. Med.* 134: 90s–108s, 1971.
7. Göze, O., Medicus, R. G. & Müller-Eberhard, H. J.: Alternative pathway of complement: nonenzymatic, reversible transition of precursor to active properdin. *J. Immunol.* 118: 525–528, 1977.
8. Kjellman, M., Laurell, A.-B., Löw, B. & Sjöholm, A. G.: Homozygous deficiency of C4 in a child with cutaneous lupus erythematosus. Unpublished results.
9. Lachman, P. J. & Hobart, M. J.: Complement technology. In: Weir, D. M. (Ed.): *Handbook of experimental immunology*, 3rd ed., Blackwell Scientific Publications, Oxford 1978, p. 5A1–5A23.
10. Laurell, A.-B., Mårtensson, U. & Sjöholm, A. G.: C1 subcomponent complexes in normal and pathological sera studied by crossed immunoelectrophoresis. *Acta path. microbiol. scand. Sect. C*, 84: 455–464, 1976.
11. Laurell, A.-B., Mårtensson, U. & Sjöholm, A. G.: Studies of C1 subcomponents in chronic urticaria and angioedema. *Int. Archs Allergy appl. Immun.* 54: 434–442, 1977.
12. Laurell, A.-B., Mårtensson, U. & Sjöholm, A. G.: The development of simple tests for C1q, C1r, C1s and C2 and the determination of properdin. In: Opferkuch, W., Rother, K. & Schultz, D. R. (Eds.): *Clinical aspects of the complement system*. Georg Thieme Publishers, Stuttgart 1978, p. 12–14.
13. Loos, M., Laurell, A.-B., Sjöholm, A. G., Mårtensson, U. & Berkel, A. I.: Immunochemical and functional analyses of a complete C1q deficiency in man: Evidence that C1r and C1s are in the native form, and that they reassociate with purified C1q to form macromolecular C1. *J. Immunol.* 124: 59–63, 1980.
14. Manni, J. A. & Müller-Eberhard, H. J.: The eighth component of human complement (C8): isolation, characterisation and hemolytic efficiency. *J. exp. Med.* 130: 1145–1160, 1969.
15. Nydegger, U. E., Fearon, D. T. & Austen, K. F.: The modulation of the alternative pathway of complement in C2-deficient human serum by changes in concentration of the component and control proteins. *J. Immunol.* 120: 1404–1408, 1978.
16. Polley, M. J. & Müller-Eberhard, H. J.: The second component of human complement: Its isolation, fragmentation by C'1 esterase and incorporation into C'3 convertase. *J. exp. Med.* 128: 535–551, 1968.
17. Rapp, H. J. & Borsos, T.: *Molecular basis of complement action*. Appleton-Century-Crofts, New York 1970.
18. Rother, U. et al. Personal communication. 1979.
19. Siccardi, A. G., Marconi, M. M., Ferrari, F. A., Fortunato, A., Gianetti, A., Sacchi, F. T., Beretta, A., Ugazio, A. G. & Jayakar, S. D.: High incidence and heterogeneity of functional defects of the alternative pathway of complement among atopic children. *J. Clin. Lab. Immunol.* 3: 165–170, 1980.
20. Sjöholm, A. G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. *Scand. J. Immunol.* 4: 25–30, 1975.
21. Soothill, J. F. & Harvey, B. A. M.: A defect of the alternative pathway of complement. *Clin. exp. Immunol.* 27: 30–33, 1977.
22. Tack, B. F. & Prahl, J. W.: Third component of human complement: purification from plasma and physicochemical characterization. *Biochemistry* 15: 4513–4521, 1976.
23. Thompson, R. A. & Rowe, D. S.: Immune haemolysis in agar: demonstration of the protective action of antibodies. *Immunology* 13: 411–420, 1967.
24. Wahn, V., Rother, U., Rauterberg, E. W. & Laurell, A.-B.: A third case of C3b inactivator deficiency. In press. *J. Clin. Invest.*
25. Yonemasu, K. & Stroud, R. M.: C1q: rapid purification method for preparation of monospecific antisera and for biochemical studies. *J. Immunol.* 106: 304–313, 1971.

Acta path. microbiol. immunol. scand. Sect. C

INHIBITION OF COMPLEMENT-MEDIATED HEMOLYSIS IN GEL
BY RHEUMATOID FACTORS

Lennart Truedsson and Anders G. Sjöholm,
Institute of Medical Microbiology, University of Lund, Lund,
Sweden.

Truedsson, L. & Sjöholm, A.G.: Inhibition of complement-mediated hemolysis in gel by rheumatoid factors.

SUMMARY

When subjected to a hemolysis in gel (HIG) assay for the detection of complement deficiency, 9 of 37 sera from patients with classical rheumatoid arthritis produced impaired lysis of sensitized sheep erythrocytes. All sera were normal in a test for the alternative pathway and no major abnormalities were found within the complement system. Using a two-step HIG technique, with guinea pig serum as the complement source, all sera were shown to inhibit lysis of sheep erythrocytes sensitized with rabbit IgG. Lysis of IgM coated erythrocytes was not inhibited. The agglutination titers in a Waaler-Rose test, and the areas of inhibition in the two-step HIG assay with IgG-sensitized erythrocytes, were correlated ($r = 0.80$, $p < 0.001$). Absorption of serum with rabbit IgG coupled to Sepharose 4B, reduced the capacity to inhibit immune hemolysis. The eluate from IgG-Sepharose contained rheumatoid factors and also inhibited immune hemolysis. The findings suggested that rheumatoid factors in serum were responsible for inhibition in the HIG assays used.

Key words: Anti-immunoglobulins; complement screening; rheumatoid factors; hemolysis in gel.

L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

INTRODUCTION

A hemolysis in gel (HIG) screening procedure for the detection of complement (C) deficiency states has been described (11). The procedure consists of one assay for the classical pathway, using sensitized sheep erythrocytes, and one assay for the alternative pathway with guinea pig erythrocytes. Some pathological sera with normal levels of C components gave aberrant patterns in the classical pathway test, frequently with a zone of unlysed cells within the lytic area (11). This occurred with various pathological sera including sera from patients with rheumatoid arthritis (RA).

In this study, rheumatoid factor (RF) positive sera from 37 patients with RA were examined for their capacity to inhibit immune hemolysis in gel.

MATERIALS AND METHODS

Sheep erythrocytes (E). Sheep blood was collected and kept in Alsever's solution as previously described (11).

Human sera. Blood samples were obtained from 37 patients with classical RF positive RA. Normal human sera (NHS) were obtained from apparently healthy donors. The sera were frozen at -80°C within 24 hours of collection. In some experiments the sera were pre-absorbed with an equal volume of packed E at 4°C for 2 h.

Reference serum. The international reference preparation of rheumatoid arthritis serum (RF-reference) was supplied by the WHO Laboratory for biological standards, Statens Seruminstitut, Copenhagen, Denmark (1).

Guinea pig serum was pooled and frozen at -80°C within 4 h of collection. Four volumes of the serum was absorbed with one volume of packed E at 4°C for 2 h, before being used as a source of hemolytic C.

Sensitization of E. Commercial rabbit amboceptor (SBL, Stockholm, Sweden) was used for sensitization of E in the classical pathway screening assay (11). The amboceptor was gel filtered to separate IgG and IgM antibodies (10). E were sensitized with IgG (EAG) or IgM (EAM). The lowest dose of antibodies, that did not limit lysis in a standard amboceptor titration (6), was used for the sensitization of erythrocytes in hemolytic tests. For agglutination, half the lowest dose of antibodies that agglutinated E was used for sensitization.

Screening assays for C deficiencies. The HIG screening procedure with sensitized E (EA) for the classical pathway, and with guinea pig erythrocytes for the alternative pathway was used (11).

Two-step HIG assay for the detection of inhibitory factors.

Serum factors inhibiting C mediated lysis of sensitized erythrocytes were detected as described earlier (10). In short, 5 μ l of the test samples, heated at 56 °C for 30 min, were applied to 3 mm diameter wells punched in agarose gels containing 1% EAG or EAM. The gels were incubated at 4 °C for 18 h, and then flooded with guinea pig serum at the highest dilution (1:2 - 1:4) that produced full hemolysis. Veronal-buffered saline with Ca^{2+} and Mg^{2+} (VBS⁺⁺) (6) was used as diluent. The agarose plates were kept at 4 °C for 2 h and then, for optimal hemolysis, at 37 °C for 2 h.

Hemagglutination tests. Twofold dilutions in 25 μ l volumes of sera heated at 56 °C for 30 min and then absorbed with E were made in microtiter plates. Equal volumes of 1 % EAG or EAM in 0.9 % NaCl were added and the plates were read after 1 h at 37 °C.

Immune absorbtion experiments. Normal rabbit IgG was purified by precipitation with polyethylene glycol 6000 (KEBO AB, Stockholm, Sweden), and by chromatography on DEAE cellulose. The IgG was coupled to cyanogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at 10 mg protein/ml gel according to the Pharmacia recommendations. 200 μ l of the test sera, absorbed with E, was passed through columns

containing 1 ml IgG-Sepharose, equilibrated with VBS⁺⁺. The sera were then concentrated to the original volume with Minicon CS 15 (Amicon Corp., Lexington, Mass., USA). Three ml of a heat inactivated serum, absorbed with E, was further absorbed on a 10 ml IgG-Sepharose column. After washing with VBS⁺⁺, the column was eluted with a buffer containing 4 mol/l MgCl₂ and 50 mmol/l Tris. The eluate was dialyzed against 0.15 mol/l NaCl and concentrated to the original volume by ultrafiltration on a PM 10 membrane (Amicon Corp. Lexington, Mass., USA).

Quantitation of C components and immunoglobulins. Electro-immunoassay was used for the determination of C1q, C1s (4), C4, C3, (7) IgG, IgA (5) and IgM (3).

Circulating immune complexes were measured by the C1q binding assay (12). The results were given as the amount of heat aggregated IgG with equal C1q binding capacity (2). Values <100 mg/l in equivalents of aggregated IgG were considered negative.

Statistical analysis. Correlations were calculated with the Spearman Rank correlation test.

RESULTS

HIG screening assay

Nine of the 37 sera tested showed clearly aberrant lytic patterns in the HIG screening assay for the classical pathway, the abnormal pattern being either a zone of unlysed cells within the lytic area, or a zone of unlysed cells adjacent to the application well (Fig. 1.B). Absorption with E eliminated the

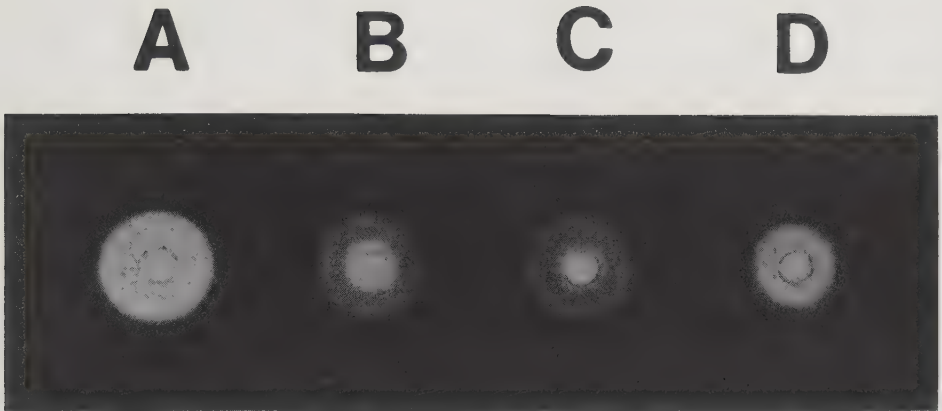


Fig. 1. HIG screening assay for the classical pathway. Normal human serum (A), RA serum (B), RA serum absorbed with E (C) and absorbed both with E and rabbit IgG-Sepharose (D). The margins of the wells are marked.

Table I. C1q, C1s, C4 and C3 in the RA sera. Values are given in per cent of normal.

	mean	range	reference area
C1q	130	74 - 360	78 - 130
C1s	140	96 - 206	79 - 139
C4	116	42 - 196	53 - 207
C3	130	69 - 187	70 - 136

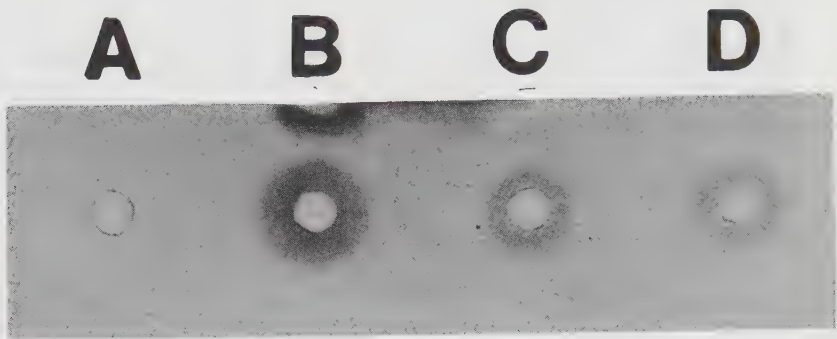


Fig. 2. Detection of factors inhibiting lysis of IgG-sensitized sheep erythrocytes by a two-step HIG technique. Normal human serum (A), RA serum , inactivated at 56 °C for 30 min and absorbed with E (B), inactivated and absorbed with E and then rabbit IgG-Sepharose (C) and the RF containing eluate from IgG-Sepharose (D). The margins of the wells are marked.

inner lytic zone, but the zone of unlysed cells remained (Fig. 1.C). After further absorption with IgG-sepharose performed with two sera, no inhibition zone was appeared (Fig. 1.D). All sera were normal in the test for the alternative pathway.

Quantitation of C components

The mean and range of the concentrations of C1q, C1s, C4 and C3 are given in Table I. None of the patients showed markedly low values and no relationship was found between the concentrations of the C components and the aberrations in the HIG test for the classical pathway.

Analysis of inhibitory factors

Factors inhibitory to immune hemolysis in the sera were demonstrated by a two-step HIG assay. Heat inactivated sera were absorbed with E before testing. In contrast to NHS, all 37 sera, and the RF-reference, inhibited immune hemolysis of EAG by guinea pig C, the zones of inhibition being clearly defined (Fig. 2). Only one serum inhibited hemolysis of EAM. The sera agglutinated EAG in titers varying from 1/16 to 1/2048. None of the sera agglutinated EAM. Agglutination titers correlated to the areas of the inhibition zones obtained with EAG in the twostep HIG assay ($r = 0.80$, $p < 0.001$) (Fig. 3).

Further analysis of the inhibitory factors was performed with two sera giving rise to abnormal patterns in the HIG screening assay. Absorption with rabbit IgG-Sepharose resulted in reduction of the inhibition zones in the two-step HIG assay with EAG (Fig. 2.C), and the agglutination titers were reduced from 1/512 and 1/2048 to $<1/4$ and 1/8, respectively. The concentrations of IgG, IgA and IgM were not significantly changed in the absorbed samples. The eluate from IgG-Sepharose, which agglutinated EAG in titer 1/128, gave inhibition of immune

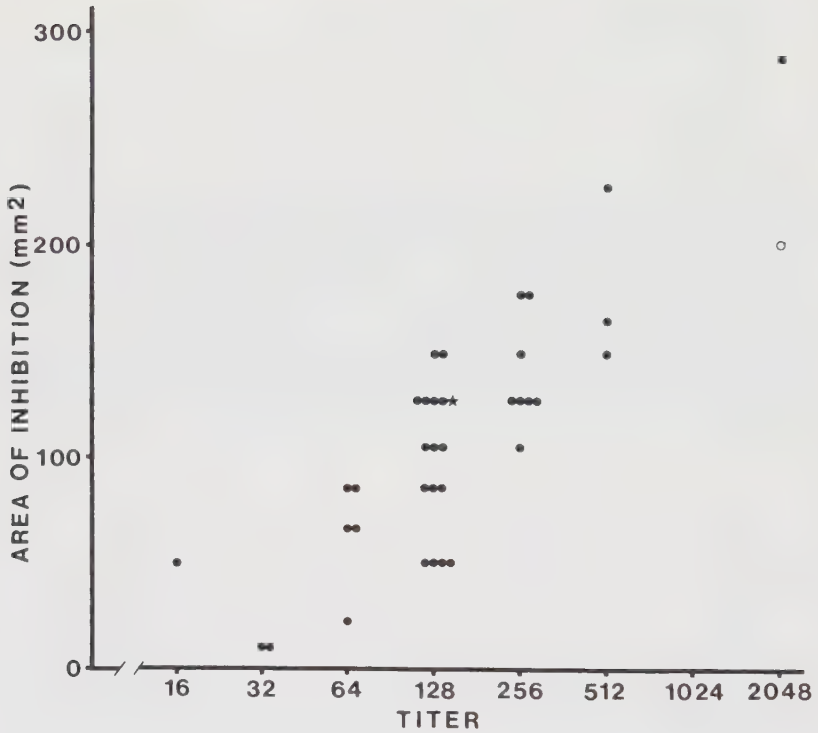


Fig. 3. Inhibition areas obtained with RA sera by a two-step HIG technique with IgG-sensitized sheep erythrocytes compared with agglutination titers with the same cells. The eluate from IgG-Sepharose, after passage of RA serum, is indicated with a star and an RA serum which caused inhibition of hemolysis of IgM-sensitized sheep erythrocytes with an open symbol. Titers are given as reciprocals of the lowest dilution causing agglutination.

Circulating immune complexes

Increased concentrations of circulating immune complexes were found in 26 of the 37 sera. Values higher than 1000 mg aggregated IgG equivalents/l were produced by ten sera, four of which impaired lysis in the HIG screening assay. One serum, containing > 4000 mg IgG equivalents/l, inhibited immune hemolysis of EAG as well as EAM. The serum agglutinated EAG in titer 1/2048, but did not agglutinate EAM. No clear correlation was found between the areas of the inhibition zones, estimated by the two-step HIG technique, and the amounts of immune complexes.

DISCUSSION

Abnormal patterns of hemolysis, unrelated to major disorders of the C system, have been observed in pathological sera on screening for C deficiency by HIG (11). IgG antibodies against rabbit IgM were recently reported to account for the inhibition of immune hemolysis by many IgA deficient sera in the screening assay (10). In the present study, where patients with RF-positive RA were investigated, 24% of the sera gave rise to patterns that suggested the presence of factors capable of inhibiting immune hemolysis. Since a mixture of rabbit IgM and IgG antibodies was used for the sensitization of sheep cells in the test, the possibility of interference by RF reacting with rabbit IgG was considered.

The RA sera consistently inhibited lysis of IgG sensitized cells in a two-step HIG assay. The inhibition areas were closely correlated with the agglutination titers of RF for IgG sensitized cells (Fig. 3). Passage of RA serum over rabbit IgG-Sepharose to absorb the RF also reduced the capacity of the

serum to inhibit immune hemolysis. In addition, the eluate (containing RF) from IgG-Sepharose also gave rise to inhibition (Fig. 2.D). With this background it was concluded that the main cause of inhibition in the HIG assays was the presence of RF in the sera.

Only one serum inhibited immune hemolysis of IgM sensitized cells. Antibodies to rabbit IgM, such as found in IgA deficiency (10), were not present but the serum contained very high concentrations of C1q binding immune complexes. With this exception there was no evidence that soluble immune complexes contributed to inhibition of C mediated HIG.

Cells coated with reduced and alkylated IgG have been used in hemolytic assays for RF activity (8, 9) which implies that RF are able to fix C. The inhibition of immune hemolysis by RF might possibly be due to a subpopulation of RF molecules that do not fix C. On the other hand, C-fixing RF bound to intact IgG on the cell surface might inhibit immune hemolysis by causing hemolytically inefficient C activation. In this case the two-step HIG assay, as described here, might provide a simple means of quantitating C-fixing RF.

Acknowledgements

This work has been supported by grants from the Swedish Medical Research Council (B83-16X-68-19C), Greta och Johan Kocks Stiftelser Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism and Alfred Österlunds Stiftelse.

REFERENCES

1. Anderson, S.G., Bentzon, M.W., Houba, V. & Krag, P.: International reference preparation of rheumatoid arthritis serum. Bull. Wld. Hlth. Org. 42:311-318, 1970.
2. Berglund, K., Laurell, A.-B., Nived, O., Sjöholm, A.G. & Sturfelt, G.: Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relations and changes on suppression of inflammation. J. Clin. Lab. Immunol. 4:7-14, 1980.
3. Grubb, A.O.: Crossed immunoelectrophoresis and electroimmunoassay of IgM. J. Immunol. 112:1420-1425, 1974.
4. Laurell, A.-B., Mårtensson, U. & Sjöholm, A.G.: The development of simple tests for C1q, C1r, C1s and C2 and the determination of properdin. In: Opferkuch, W., Rother, K. & Schultz, D.R. (Eds.): Clinical aspects of the complement system. Georg Thieme Publishers, Stuttgart 1978, pp. 12-14.
5. Laurell, C.-B.: Electroimmunoassay. Scand. J. Clin. Lab. Invest. 29, Suppl. 124, 1972, pp. 21-37.
6. Rapp, H.J. & Borsos, T. : Molecular basis of complement action. Appleton-Century-Crofts, New York 1970.
7. Sjöholm, A.G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand. J. Immunol. 4:25-30, 1975.

8. Tanimoto, K., Cooper, N.R., Johnson, J.S. & Vaughan, J.H.: Complement fixation by rheumatoid factor. *J. Clin. Invest.* 55:437-445, 1975.
9. Taylor-Upsahl, M.M., Johnson, P.M., Mellbye, O.J. & Natvig, J.B.: A study of complement fixation by rheumatoid factor using a haemolytic assay system. *Clin. exp. Immunol.* 28: 204-211, 1977.
10. Truedsson, L., Axelsson, U. & Laurell, A.-B.: Frequent occurrence of anti-rabbit IgM in IgA deficiency. *Acta path. microbiol. immunol. scand. Sect. C*, 90:315-320, 1982.
11. Truedsson, L., Sjöholm, A.G. & Laurell, A.-B.: Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. *Acta path. microbiol. scand. Sect. C*, 89:161-166, 1981.
12. Zubler, R.H., Lange, G., Lambert, P.H. & Miescher, P.A.: Detection of immune complexes in unheated sera by a modified ¹²⁵I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. *J. Immunol.* 116:232-235, 1976.

FREQUENT OCCURRENCE OF ANTI-RABBIT IgM IN IgA DEFICIENCY

L. TRUEDSSON¹, U. AXELSSON² and A.-B. LAURELL¹

¹Institute of Medical Microbiology, University of Lund, Sweden and ²Department of Medicine, Karlstad, Sweden

Truedsson, L., Axelsson, U. & Laurell, A.-B. Frequent occurrence of anti-rabbit IgM in IgA deficiency. *Acta path. microbiol. immunol. scand. Sect. C, 90: 315–320, 1982.*

Analysis of IgA deficient sera revealed impaired hemolysis of sensitized sheep erythrocytes when tested by a hemolysis in gel (HIG) assay developed for detection of complement deficiencies. All sera were normal in a test for the alternative pathway. The impaired hemolysis was not related to complement aberrations but was caused by antibodies to rabbit IgM, demonstrated in 14 of 21 IgA deficient sera, by use of HIG technique and by agglutination. The presence of these antibodies was not related to age, sex or disease. One serum was further examined and the antibodies were shown to be of IgG class.

Key words: Hemolysis in gel; IgA deficiency; anti-immunoglobulins.

L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 27.v.82 Accepted 17.vi.82

Selective IgA deficiency is the most common immunodeficiency with a frequency of 0.2–0.3 % (2). IgA deficiency is often associated with significant disease (1, 6), but the disorder also occurs in clinically healthy individuals (1, 18). It is associated with the presence of certain immunological abnormalities such as antibodies against human IgG, human IgA, bovine IgM, human thyroglobulin and various tissue antigens (1).

IgA deficient sera were frequently found to give atypically impaired lytic reactions in a hemolysis in gel (HIG) assay (25), used to detect deficiencies within the complement (C) system. In this study the sera were shown to contain antibodies against rabbit IgM, inhibitory to C mediated lysis. Some characteristics of the antibodies and relations to other immunological laboratory parameters are reported.

MATERIALS AND METHODS

IgA deficient sera. Selective IgA deficiency was defined as serum IgA concentrations of less than 0.05 g/l with normal or increased levels of IgG and IgM in serum.

Serum samples from 21 human subjects with selective IgA deficiency were studied. Twelve of the individuals (group A), had been subjectively healthy for the last 15 years, whereas the other 9 (group B) suffered from recurrent infections and various diseases. Blood samples were centrifuged at room temperature and frozen at –80 °C within four hours of collection. In group B four samples were stored at –20 °C. Table 1 lists age, sex, diagnosis and laboratory data for the IgA deficient individuals.

Normal human sera. Serum samples from blood donors and from 13 apparently healthy individuals age-matched to the clinically healthy IgA deficient group were collected and handled as described above.

Absorption. In some of the experiments sera were absorbed with an equal volume of packed, not sensitized, sheep red cells at 4 °C for two hours.

Sensitization of sheep red blood cells (E). Rabbit anti-sheep red blood cell serum (SBL, Stockholm, Sweden) was gel filtered on Sephacryl S300 (Pharmacia, Uppsala, Sweden). The fractions agglutinating E, containing IgG and IgM respectively, were pooled. E were sensitized with antibodies of IgG (EAG) or IgM class (EAM). The lowest dose of antibodies, that did not limit lysis in a standard amboceptor titration, was used for sensitization (17).

Functional C assays. CH50 was determined according to *Rapp & Borsos* (17) using EAM.

Sera were tested with the HIG assay previously described (25), using sensitized E (EA) for the classical pathway and guinea pig erythrocytes for the alternative pathway.

Modifications of the test for analysis of the classical pathway with 1% EAG or EAM in the gel were also used.

HIG assay for detection of inhibitory factors. Agarose plates, containing 1% EAG and EAM respectively, were prepared by methods previously described (25). Five μ l of the sera, heated at 56 °C for 30 min, were applied to wells of 3 mm diameter and the plates were incubated in a moist chamber at 4 °C for 18 hours. As a C source, filter papers with 4 ml fresh guinea pig serum (GPS) were placed on top of the gels (205 $\times$ 105 $\times$ 1 mm) and left on the plates for two hours at 4 °C. Following incubation at 37 °C for two hours, the filter papers were removed. The plates were preserved as described

previously (25). The area of inhibited lysis was measured as the difference between the area enclosed by the outer border of the inhibition zone and the area of the well.

Hemagglutination tests. Twofold dilutions in 25 μ l volumes of sera heated at 56 °C for 30 min absorbed with E were made in microtitre plates. Equal volumes of 1% EAG or EAM in 0.9% NaCl were added and the plates were read after one hour at 37 °C.

Agarose electrophoresis combined with HIG. Sera from IgA deficient individuals were subjected to electrophoresis according to described methods (8) with addition of 0.15 M sucrose to the gel buffer. Agarose strips 10 mm wide were cut out of the gel after electrophoretic separation and placed on top of a gel containing EAM. After incubation at 4 °C for two hours the strips were removed and hemolysis developed with GPS as described above.

Absorption with protein A-Sepharose CL-4B. Protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden), 0.5 ml, was incubated with 0.5 ml serum for 20 min at room

TABLE 1. *Summary of Data on 21 IgA Deficient Subjects, 12 Clinically Healthy and 9 with Significant Disease*

Subject Diagnosis	Age (yr)	Sex	Serum IgG (g/l)	Waller-Rose test (titre)	Agglutination of EAM (titre)	C-function ^a , classical pathway
<i>Group A</i>						
K.A. Normal	45	M	14.0	Neg ^b	Neg ^c	N ^d
B.B. Normal	67	F	14.9	1/128	1/64	Impaired lysis
L.E. Normal	53	M	11.8	1/128	1/256	No lysis
A.G. Normal	67	F	18.2	1/32	1/64	Impaired lysis
A.H. Normal	65	F	15.8	Neg	Neg	N
E.J. Normal	62	M	17.1	Neg	Neg	N
P.K. Normal	90	M	19.3	Neg	Neg	N
H.L. Normal	58	M	17.7	1/8	1/4	Impaired lysis
Ho.L. Normal	60	M	18.4	1/128	1/128	No lysis
G.N. Normal	66	M	20.4	1/64	1/128	No lysis
M.M. Normal	69	F	17.7	1/32	1/128	Impaired lysis
S.N. Normal	55	M	14.3	1/128	1/256	Impaired lysis
<i>Group B</i>						
A.L. Rheumatoid arthritis	56	F	16.2	1/32	1/32	No lysis
F.A. Recurrent infections	9	M	18.0	Neg	Neg	N
T.A. Recurrent infections	5	M	11.3	Neg	Neg	N
H.M. Recurrent infections, allergies		F	14.6	1/16	Neg	N
M.H. Recurrent infections	11	M	13.2	1/32	1/16	Impaired lysis
Be.B. HLA-B27 associated arthritis	46	M	30.0	Neg	1/4	Not tested
S.O. Recurrent infections, allergies	10	M	11.5	1/16	1/4	Not tested
K.N. Recurrent infections	25	M	8.2	1/32	1/16	Not tested
A.P. Hepatopathia, psoriasis	53	M	18.2	1/128	1/64	Not tested

^a C-function estimated with the screening method (25)

^b Titres < 1/8

^c Titres < 1/4

^d N stands for normal or very slightly impaired lysis.

temperature. Four ml veronal-buffered saline with Ca^{2+} and Mg^{2+} (VBS^{2+}) (25) was added. After stirring and centrifugation the supernatant was removed. The Sepharose was washed 3 times with VBS^{2+} and then treated with 4.5 ml 0.2 M glycine buffer, pH 3.0 for 20 min at room temperature. After centrifugation the supernatant was collected and dialyzed against VBS^{2+} overnight.

Radioactive labeling of rabbit anti-human IgG. Rabbit anti-human IgG (DAKOPATTS a/s, Copenhagen, Denmark) was labelled with ^{125}I using chloramine-T(15).

Binding of ^{125}I anti-human IgG to EAM. EAM or E, 1% in VBS^{2+} , 0.8 ml, were incubated at 37°C for 45 min with 0.16 ml of serial dilutions of heat inactivated serum absorbed with E. After washing, 0.2 ml of the cell suspension was mixed with $20\ \mu\text{l}$ ^{125}I anti-human IgG at appropriate concentration in VBS^{2+} and incubated at 37°C for 45 min. Uptake of the label to the cells was determined by application of the technique described by Sobel *et al.* (22).

Immunoglobulin quantitations. IgG, IgA and IgM were determined by electroimmunoassay (13, 5). Normal ranges for adults are for IgG 6.5–15.0 g/l and for IgM 0.3–2.0 g/l. The values given by Johansson & Berg (9) were used as normal ranges for immunoglobulin levels in children.

Quantitation of C components. C1q, C1s, C2, C4, C3 and factor B were quantitated by electroimmunoassay (12, 21).

Antinuclear antibodies were determined by indirect immunofluorescence technique (16).

Waalser-Rose test was performed essentially as described by Ball (3) using cells coated with whole rabbit anti-sheep red blood cell serum (SBL, Stockholm, Sweden).

Latex fixation test (20) was performed using a commercial screen test (RA-TEST, Hyland Diagnostics, IL, USA).

Circulating immune complexes were measured by the C1q binding assay (27). The results were given as the amount of heat aggregated IgG with equal C1q binding capacity (4). Values ≤ 100 mg IgG equivalents/l were considered as negative.

Statistical analysis. The Spearman rank correlation test was used for calculation of correlations.

RESULTS

Screening for C Deficiencies by HIG

The four sera stored at -20°C were not included in these analysis. Ten of the 17 sera tested, 8 belonging to group A and 2 to group B, showed aberrant lytic patterns in the HIG test with sheep cells sensitized with a mixture of antibodies of IgG and IgM class (Table 1). In contrast, the 13 sera from healthy individuals, age-matched with group A, showed normal lysis. All sera were normal in the test for the alternative pathway.

Of the 10 aberrant sera, 4 gave no lysis or a very small lytic zone next to the application well. Six sera

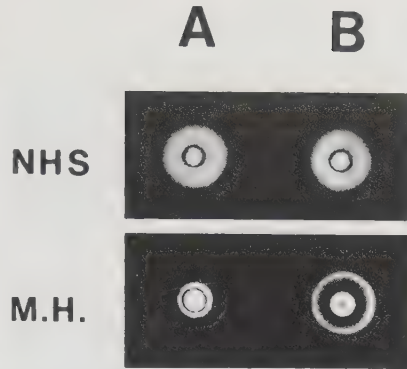


Fig. 1. Results with the HIG screening assay for C deficiencies obtained with IgA deficient serum (M.H.) and normal human serum (NHS). The figure shows the lysis patterns obtained with EA using fresh serum (A) and fresh serum absorbed with E (B).

gave rise to a lytic pattern consisting of a zone of unlysed cells, surrounded by an outer lytic zone and usually by an inner zone of lysed cells as well. Absorption with E removed the inner lytic zone, leaving the zone of unlysed cells (Fig. 1).

All the IgA deficient sera tested showed normal lytic zones in the modified test for the classical pathway with EAG in the gel, while the abnormal lytic patterns remained with EAM.

The CH50 was determined for 14 sera. The sera showing inhibition in HIG tests did not deviate from the sera showing normal lysis in these tests (Fig. 2)

Quantitation of C Components

The values obtained for the individual C components investigated were mainly within normal

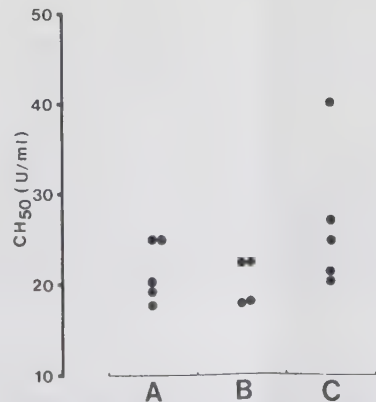


Fig. 2. CH50 values using EAM obtained with normal human sera (A), IgA deficient sera with impaired lysis in the HIG screening assay (B) and IgA deficient sera with normal HIG assay (C).

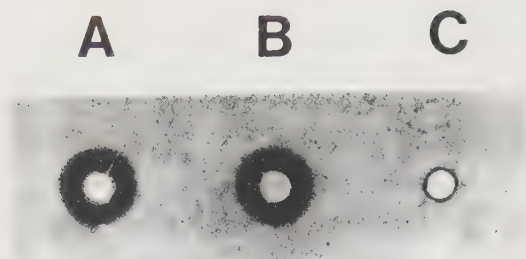


Fig. 3. Detection of factors inhibiting lysis of EAM by HIG technique in one IgA deficient serum (A.L.). (A), inactivated at 56 °C for 30 min, (B), inactivated and absorbed with E and (C), inactivated and absorbed with EAM.

ranges. No relationship between the levels of C factors and the lysis inhibition of EAM was found.

Analysis of Inhibitory Factors

Prior to testing, heat inactivated sera were absorbed with E. Fourteen of the 21 sera inhibited lysis of EAM induced by guinea pig C, including the 10 sera with aberrant HIG screening assay and the four sera not tested for C function. None of the 13 control sera caused inhibition. The capacity to inhibit lysis was not related to age or sex and its frequency was 67% in both groups with IgA deficiency. The diameters of the inhibition zones varied between 5.5 and 9.5 mm.

The inhibition of lysis disappeared after absorption of sera with EAM, though not after absorption with E (Fig. 3).

Only one serum (Be.B.), inhibitory to lysis of EAM, also inhibited lysis of EAG.

All sera inhibiting lysis of EAM were found to

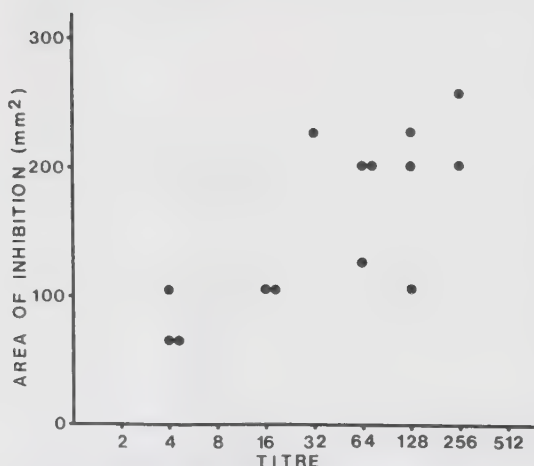


Fig. 4. Inhibition areas obtained with IgA deficient sera by HIG technique with EAM compared with agglutination titres with EAM. Titres are given as the reciprocals of the lowest dilution causing agglutination.

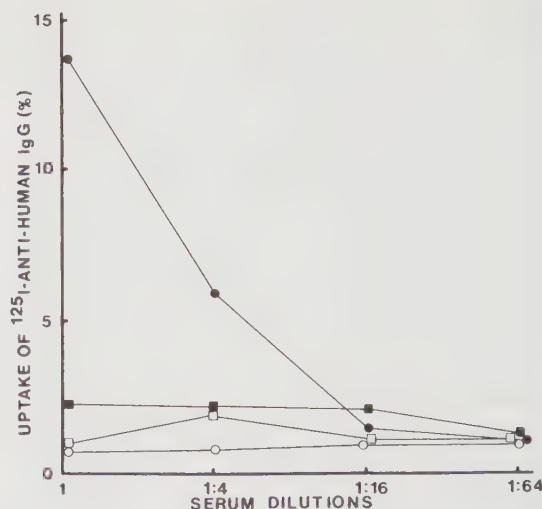


Fig. 5. Uptake of ^{125}I anti-human IgG to EAM incubated with IgA deficient serum (A.L.) or normal human serum (NHS). (●) EAM + A.L., (○) EAM + NHS, (■) E + A.L., (□) E + NHS.

agglutinate EAM cells i titres between 1/4 and 1/256 (Table 1). The titres correlated to the inhibition areas obtained with EAM in the gel ($r = 0.74$, $p < 0.01$) (Fig. 4). No agglutination was obtained with EAG (titres $< 1/4$).

Sera causing inhibition of lysis of EAM were investigated with agarose electrophoresis combined with HIG. In all cases inhibition of lysis appeared in a broad area of the γ -region.

Further analysis of the inhibiting factor was performed with one serum (A.L.). The lysis-inhibiting factor was no longer demonstrable after absorption with protein A. About 50% of the original inhibitory capacity was found in the eluate from protein A as measured by the HIG assay.

Serum A.L., inactivated and absorbed with E, was incubated with EAM; after washing, the cells were incubated with ^{125}I anti-human IgG. An uptake of 13.5% of the label was obtained (Fig. 5). Controls with E caused a maximum uptake of 2.5%, and with normal human serum no difference was noted on incubation with either EAM or E.

Other Laboratory Findings

Twelve of the IgA deficient sera had increased levels of IgG. One serum (A.P.) had an increased level of IgM. The levels of IgG and IgM were not correlated to the size of the inhibition zones.

Waller-Rose test titres between 1/4 and 1/128 were found in 14 sera (Table 1). The agglutination titres obtained with EAM correlated to the Waller-Rose test titres ($r = 0.83$, $p < 0.01$).

The latex fixation test was negative for the IgA deficient sera.

Antinuclear antibodies could not be detected in any of the sera investigated.

Circulating immune complexes were found neither in group A nor in 4 of the sera belonging to group B. Two sera in group B had clearly elevated levels of circulating immune complexes, 350 mg IgG equivalents/l and 1400 mg IgG equivalents/l, respectively. The amounts of circulating immune complexes were not correlated to the lysis inhibition zones.

DISCUSSION

In earlier work with pathological sera, using a hemolysis in gel (HIG) method for the screening of C factor deficiencies, various patterns of impaired hemolysis were observed (25). The present study gave evidence that 10 of 17 sera from subjects with selective IgA deficiency behaved abnormally in the test for the classical pathway due to the presence of antibodies inhibiting lysis. The 13 age-matched control human sera, were normal in agreement with earlier studies (25).

The abnormal patterns found were either absence of hemolysis, or a zone of unlysed cells within the lytic area. With most sera a narrow lytic zone appeared adjacent to the application well. *Thompson & Rowe* (23) reported that rarely occurring antibodies to E, can inhibit lysis of EA. Since absorption with E did not remove the inhibition zone (Fig. 1, 3), such antibodies could not be responsible for the inhibition observed here. In a HIG system using E, *Romeyn et al.* (19) have noted the presence of an inner lytic zone, caused by Forssman antibodies often present in human sera. Such antibodies caused the inner lytic zone nearest the well, since after absorption of fresh serum with E in the cold, this lytic zone disappeared (Fig. 1).

In the present study, IgA deficient sera showing an abnormal HIG pattern, inhibited lysis of EAM but with one exception, not lysis of EAG. The inhibition zones disappeared after absorption of sera with EAM, indicating that the inhibitory factor reacts with the sensitizing IgM antibody (Fig. 3). Sera causing inhibition also agglutinated EAM, but not EAG. The agglutination titres with EAM were correlated to the inhibition areas as well as to the titres in the Waaler-Rose test (Fig. 4, Table 1). The lysis inhibiting factor was located in the γ -region. These results suggest that antibodies to rabbit IgM were responsible for the inhibition of lysis.

The inhibitory factor in serum from a patient (A.L.) with sero-negative rheumatoid arthritis, was given further study. The inhibitory factor in this

serum was bound to protein A, showing that antibodies against rabbit IgM of IgG subclass 1, 2 or 4 (11) were responsible for the lysis inhibition. This was also supported by the finding of an increased uptake of ^{125}I anti-human IgG antibodies on EAM after incubation of the cells with this serum (Fig. 5). The mechanism by which these antibodies inhibit the lytic reaction was not examined further in the present study.

In the CH50 test the effect of such antibodies on C mediated lysis will hardly be apparent, even if IgM is used for sensitization of the cells (Fig. 2). This is probably due to the dilution of sera to 1/50 before determination of the CH50 titre, in contrast to the HIG assay where undiluted sera are investigated.

Precipitating antibodies to bovine IgM in sera from IgA deficient humans have been reported (7, 14, 24) and in two studies the precipitating antibodies did not react with rabbit or human IgM (7, 24). *Wells et al.* (26) found antibodies to human IgM in IgA deficient sera using a hemagglutination method. The absence of antibodies to rabbit IgM in the studies cited (7, 24), might be caused by the different techniques used. However, *Johnson et al.* (10) have reported on one IgA deficient patient with an IgG antibody reacting with human as well as rabbit IgM.

Presence of the antibodies to rabbit IgM in 14 of 21 IgA deficient individuals described here was unrelated to sex, age or disease. The mechanism causing the development of antibodies to foreign or human IgM is not known. Oral immunization facilitated by the mucosal antibody defect accompanying IgA deficiency has been proposed as an explanation of the antibodies formed against bovine IgM (7, 14, 24). Another possibility for an immunogenic stimulus might be some intestinal microorganism, sharing antigenic determinants with IgM, favored because of the IgA deficiency. Further study is needed to elucidate the reaction of the anti-rabbit IgM antibodies described here with IgM from other species.

This study shows that the HIG screening assay developed for revealing C deficiencies may also provide further information, as stressed earlier (25). The high frequency in IgA deficiency of abnormal lytic patterns in this assay was shown to depend on the presence of antibodies to rabbit IgM, and not on C factor disturbances.

This work was supported by grants from the Swedish Medical Research Council (B82-16X-68), *Föreningen för Bistånd åt Vanföra i Skåne, Greta och Johan Kocks Stiftelser, Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism and Alfred Österlunds Stiftelse.*

REFERENCES

1. *Ammann, A. J. & Hong, R.*: Selective IgA deficiency: presentation of 30 cases and a review of the literature. *Medicine* 50: 223-236, 1971.
2. *Bachmann, R.*: Studies on the serum γ A-globulin level. III. The frequency of A- γ A-globulinemia. *Scand. J. Clin. Lab. Invest.* 17: 316-320, 1965.
3. *Ball, J.*: Serum factor in rheumatoid arthritis agglutinating sensitised sheep red cells. *Lancet* 2: 520-524, 1950.
4. *Berglund, K., Laurell, A.-B., Nived, O., Sjöholm, A. G. & Sturfelt, G.*: Complement activation, circulating C1q binding substances and inflammatory activity in rheumatoid arthritis: relations and changes on suppression of inflammation. *J. Clin. Lab. Immunol.* 4: 7-14, 1980.
5. *Grubb, A. O.*: Crossed immunoelectrophoresis and electroimmunoassay of IgM. *J. Immunol.* 112: 1420-1425, 1974.
6. *Hanson, L. Å. & Brandtzaeg, P.*: The mucosal defence system. In: *Stiehm, R. T. & Fulginiti, V.* (Eds.): Immunologic disorders in infants and children. 2d. ed. W. B. Saunders, Philadelphia 1980, pp. 137-164.
7. *Huntley, C. C., Robbins, J. B., Lyerly, A. D. & Buckley, R. H.*: Characterization of precipitating antibodies to ruminant serum and milk proteins in humans with selective IgA deficiency. *New Engl. J. Med.* 284: 7-10, 1971.
8. *Johansson, B. G.*: Agarose gel electrophoresis. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124, 1972, p. 7-19.
9. *Johansson, S. G. O. & Berg, T.*: Immunoglobulin levels in healthy children. *Acta Paediat. Scand.* 56: 572-579, 1967.
10. *Johnson, P. M., Reading, C. A., Holborow, E. J. & Holdstock, D. J.*: False-positive Rose-Waaler rheumatoid factor titre given by anti-ruminant IgM antibody. *Vox. Sang.* 31: 451-455, 1976.
11. *Kronvall, G. & Williams Jr. R. C.*: Differences in anti-protein A activity among IgG subgroups. *J. Immunol.* 103: 828-833, 1969.
12. *Laurell, A.-B., Mårtensson, U. & Sjöholm, A. G.*: The development of simple tests for C1q, C1r, C1s and C2 and the determination of properdin. In: *Opferkuch, W., Rother, K. & Schultz, D. R.* (Eds.): Clinical aspects of the complement system. Georg Thieme Publishers, Stuttgart 1978, pp. 12-14.
13. *Laurell, C.-B.*: Electroimmunoassay. *Scand. J. Clin. Lab. Invest.* 29, Suppl. 124, 1972, p. 21-37.
14. *Leikola, J. & Vyas, G. N.*: Human antibodies to ruminant IgM concealing the absence of IgA in man. *J. Lab. Clin. Med.* 77: 629-638, 1971.
15. *McConahey, P. J. & Dixon, F. J.*: A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy* 29: 185-189, 1966.
16. *Nakamura, R. M.*: Laboratory tests for the evaluation of Systemic Lupus Erythematosus and related collagen disorders. In: *Nakamura, R. M.* (Ed.): Immunopathology. Clinical laboratory concepts and methods. Little, Brown and Company, Boston 1974, pp. 247-288.
17. *Rapp, H. J. & Borsos, T.*: Molecular basis of complement action. Appleton-Century-Crofts, New York 1970.
18. *Rockey, J. H., Hanson, L. Å., Heremans, J. F. & Kunkel, H. G.*: Beta-2A-aglobulinemia in two healthy men. *J. Lab. Clin. Med.* 63: 205-212, 1964.
19. *Romeyn, J. A., Baragar, F. D. & Cook, J.*: Anti-immunoglobulin analysis by diffusion patterns of inhibition and facilitation of complementary lysis in agar. III. Anti-immunoglobulin diffusion-lysis patterns of normal human and rheumatoid sera. *J. Immunol. Methods.* 7: 47-64, 1975.
20. *Singer, J. M. & Plotz, C. M.*: The latex fixation test. I. Application to serologic diagnosis of rheumatoid arthritis. *Amer. J. Med.* 21: 888-892, 1956.
21. *Sjöholm, A. G.*: Complement components in normal serum and plasma quantitated by electroimmunoassay. *Scand. J. Immunol.* 4: 25-30, 1975.
22. *Sobel, A. T., Bokisch, V. A. & Müller-Eberhard, H. J.*: C1q deviation test for the detection of immune complexes, aggregates of IgG, and bacterial products in human sera. *J. Exp. Med.* 142: 139-150, 1975.
23. *Thompson, R. A. & Rowe, D. S.*: Immune haemolysis in agar: demonstration of the protective action of antibodies. *Immunology* 13: 411-420, 1967.
24. *Tomasi, T. B. & Katz, L.*: Human antibodies against bovine immunoglobulin M in IgA deficient sera. *Clin. exp. Immunol.* 9: 3-10, 1971.
25. *Truedsson, L., Sjöholm, A. G. & Laurell, A.-B.*: Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. *Acta. path. microbiol. scand. Sect. C*, 89: 161-166, 1981.
26. *Wells, J. V., Bleumers, J. F. & Fudenberg, H. H.*: Human anti-IgM iso-antibodies in subjects with selective IgA deficiency. *Clin. exp. Immunol.* 12: 305-313, 1972.
27. *Zubler, R. H., Lange, G., Lambert, P. H. & Miescher, P. A.*: Detection of immune complexes in unheated sera by a modified 125 I-C1q binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. *J. Immunol.* 116: 232-235, 1976.

COMPLEMENT ACTIVATING RHEUMATOID FACTORS IN RHEUMATOID ARTHRITIS
STUDIED BY HAEMOLYSIS IN GEL: RELATION TO ANTIBODY CLASS AND
RESPONSE TO TREATMENT WITH PODOPHYLLOTOXIN DERIVATIVES

Lennart Truedsson, Anders G. Sjöholm and Gunnar Sturfelt

From the Section of Clinical Immunology, Institute of Medical Microbiology, University of Lund and the Department of Rheumatology, University Hospital, Lund, Sweden.

Correspondence: Dr. L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

SUMMARY

Complement (C) activating rheumatoid factors (RF) were measured in 16 patients with rheumatoid arthritis (RA) by a simple haemolysis in gel (HIG) assay. IgM-RF, but not IgG-RF or IgA-RF, measured by an enzyme linked immuno-sorbent assay, were closely correlated with C activating RF ($r=0.86$). In neither assay did the concentrations of RF appear to be directly related to C1 activation as expressed by serum concentrations of C1r-C1s-C1 inactivator complexes or of C4. In the sera, C1q binding substances, measured by C1q binding assay, were markedly correlated with C activating RF ($r=0.88$), whereas C1q binding substances detected by the C1q deviation test were not.

Treatment with podophyllotoxin derivatives for 6 months clearly reduced patients' RF concentrations. The decrease of IgG-RF, IgA-RF and IgM-RF was significantly more pronounced than that of the IgG, IgA and IgM concentrations, respectively.

Key words: Rheumatoid arthritis, rheumatoid factors, haemolysis in gel, complement activation, enzyme immunoassay, podophyllotoxin derivatives.

INTRODUCTION

Rheumatoid factors (RF) in blood and in the synovial fluid of affected joints is an important serological feature of rheumatoid arthritis (RA) (1). Complement (C) fixation by polyclonal IgM-RF has been demonstrated by means of haemolytic assay systems (2, 3). RF of IgG, IgM and possibly IgA class are considered to be of pathogenetic significance in RA. Immune complexes containing RF may activate the C system and be involved in the development of synovitis as well as of extra-articular complications (4-6).

The classic routine methods for measuring RF, the Waaler-Rose and the latex fixation tests, are based on agglutination which particularly facilitates detection of IgM antibodies. In the present study a simple haemolysis in gel (HIG) assay for measurement of RF with C activating capacity is described. Recently we reported that RF could be detected by inhibition of immune haemolysis in a slightly different HIG system (7). To permit the separate measurement of RF of different immunoglobulin classes, an enzyme linked immunosorbent assay (ELISA) was also developed.

As an extension of an earlier study on C1 activation and C1q binding immune complexes in RA, C activating RF were measured in the sera from a group of RA patients treated with podophyllotoxin derivatives (PTD) (8). C activating and class-specific RF were examined in relationship to the findings in the previous study.

MATERIALS AND METHODS

Samples

Serum and EDTA plasma samples from the patients, and from healthy blood donors were stored in aliquots at -80°C . With few exceptions, serum was used in the measurement of RF. Samples to be tested in haemolytic assays were heated at 56°C for 30 min and absorbed at 4°C for 1 h with 1/2 volume packed sheep erythrocytes (E).

Reference sera

A serum with a high RF titre (1/4096 in the Waaler-Rose test) was selected from samples routinely analysed at the laboratory, and used as reference (RF-ref) in the RF assays. The RF-ref was calibrated against the international reference preparation of rheumatoid arthritis serum supplied by the WHO Laboratory for biological standards, Statens Seruminstitut, Copenhagen, Denmark (9).

Haemolytic assays for C activating RF

Reagents. An antiserum to E was raised in rabbits. IgG antibodies were prepared by precipitation with 33 % (w/v) $(\text{NH}_4)_2\text{SO}_4$ and further purified by chromatography on DEAE-Trisacryl M (LKB-Produkter AB, Sweden).

Reduced and alkylated rabbit IgG antibodies to E were prepared by anaerobic reduction as described by Johnson and Hoffmann (10). After treatment, lytic and agglutinating titres were measured (3). Reduction and alkylation resulted in an at least 10-fold decrease of the lytic titre, while the

agglutination titre remained unchanged.

E were sensitized with reduced and alkylated rabbit IgG antibodies (EAGr) by incubation of the cells suspended in veronal buffered saline with Mg^{2+} and Ca^{2+} (VBS $^{++}$) (11) with the highest non-lytic dose of antibodies.

Guinea pig serum pre-absorbed with 1/4 volume packed E at 0 °C for 2 h was used as a source of C.

HIG assay for C activating RF. Agarose plates (205x105x1 mm) containing 0.75 % (v/v) EAGr with addition of 0.1 % gelatin were prepared as previously described (12). Seven μ l of the test samples were applied to 3 mm diameter wells in the gel. Trials with different incubation times at 4 °C, showed 64 h to be optimal. After diffusion in the cold, filter papers with 4 ml guinea pig serum diluted 1/2 in VBS $^{++}$ were placed on top of the gel and left in place for 2 h at 4 °C. After further incubation at 37 °C for 2-3 h, to ensure full immune haemolysis, the filter papers were removed. The plates with the haemolytic zones were preserved as described earlier (12). The diameters of the lytic zones were measured using a digitizer (HPAD Digitizer, Houston Instrument). Each plate included a standard, consisting of RF-ref, undiluted and in two-fold dilutions in VBS $^{++}$ up to 1/512. The sera were tested undiluted or at a 1/10 dilution in VBS $^{++}$. A reference curve was made by plotting the diameters of the lytic zones of the RF-ref against the corresponding dilutions (Fig. 1). The concentrations of C activating RF were expressed in arbitrary units (AU), referring to the RF-ref which was said to contain 1,000 AU/ml.

Haemolytic tube assay for RF. The assay for haemolytic RF with EAGr was performed essentially as described by Tanimoto et al. (2). In short, 40 μ l of the test sample in dilution 1/128 was

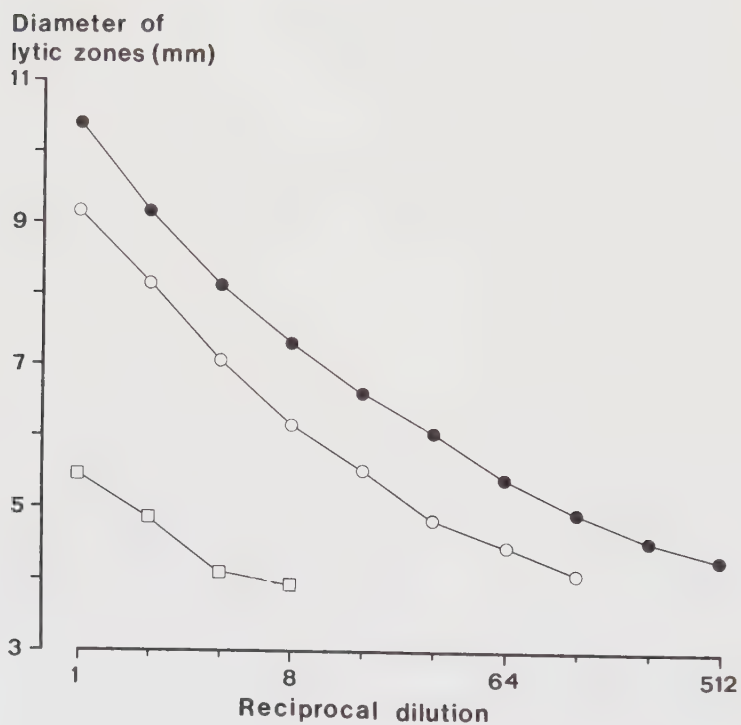


Fig. 1. Dose-response curves obtained in the haemolysis in gel assay for C activating RF. (●) the local RF-reference serum, (○) serum from a patient with rheumatoid arthritis and (□) RF containing eluate from rabbit IgG-Sepharose.

mixed with 200 μ l EAGr (2×10^8 per ml) and incubated at 37 °C for 1 h. After washing with VBS⁺⁺, the cells were suspended in guinea pig serum diluted 1:20 in VBS⁺⁺ and again incubated at 37 °C for 1 h. The lytic reaction was stopped by adding of 1 ml ice-cold VBS with 20 mmol/l EDTA. Results were given in per cent of the cells lysed.

Two-step HIG assay for detection of inhibitory factors (RF)

Serum factors inhibitory to C mediated lysis of IgG sensitized E in gel, mainly RF, were detected as described earlier (7).

ELISA for IgG-RF, IgA-RF and IgM-RF

Reagents. Antiserum to human serum albumin (HSA) (Sigma Chemicals, Mo., USA) was raised in rabbits. An IgG fraction (IgG anti-HSA) was obtained by gel filtration on AcA 44 (LKB-Produkter AB, Sweden).

Rabbit antibodies to human α , γ and μ chains (DAKO Immunoglobulins A/S, Denmark) were digested with pepsin (Millipore Corp., Freehold, N.J., USA), and F(ab')₂ fragments were isolated by gel filtration on Sephacryl S300 (Pharmacia, Sweden) (13). Analysis with SDS-PAGE (14) showed no heavy chains. F(ab')₂ fragments were conjugated with phosphatase alkaline from bovine intestine, type VII-S (Sigma Chemicals, Mo., USA) according to Voller et al., (15).

Test procedure. 1. 100 μ l of HSA, 20 mg/l in phosphate buffered saline with Na₂N₃ (PBS) (15) was added to each well of a 96 well micro-titre plate (M129B, Dynatech AG, Switzerland) and left to adsorb at 20 °C for 2 h. The plate was then washed three times with PBS.

2. For blocking of nonspecific binding, 200 μ l of a solution containing 0.1 % (w/v) gelatin (Difco lab., Mich., USA) in PBS with addition of 0.05 % (v/v) Tween 20 (PBS-T) was added to each well. After 18 h at 20 °C the plate was washed three times with PBS-T.

3. For each sample three wells were used, two of them coated with 100 μ l of an appropriate dilution of IgG anti-HSA in PBS-T, and the third left coated with HSA (control antigen). The antibodies were allowed to absorb for 2 h at 20 °C after which the plates were emptied. At this stage, plates could be frozen at -20 °C and stored for at least two months. Before use the wells were washed as in step 2.

4. Samples to be tested were diluted in PBS-T and 100 μ l of the samples were added to each well. The plates were incubated for 2 h at 20 °C and then washed as in the previous step.

5. 100 μ l of alkaline phosphatase conjugated F(ab')₂ fragments of rabbit anti-human α , γ and μ chains, suitably diluted with PBS-T, were added to each well. After 18 h at 20 °C, the plates were washed three times with PBS-T, residual PBS-T being removed by inverting the plates and patting them against paper towels.

6. 100 μ l of a substrate solution containing 1 g/l of P-nitro-phenyl phosphate (Sigma Chemicals, Mo., USA) in 10 % (v/v) diethanolamine buffer, pH 9.5 was added to each well. After 60 min at 20 °C, the enzyme activity was measured in a Titertek Multiskan photometer (Flow Laboratories) at 405 nm, giving an absorbance of approximately 1.0 for the RF-ref in dilution 1/800. In each plate the RF-ref in dilution 1/800 was added to 4 wells coated with IgG anti-HSA, and to 4 wells coated with the control antigen, HSA alone. Test samples were usually examined in dilution 1/100.

Calculation of ELISA results. Arbitrary ELISA units (AU) were

calculated in the following manner: The relative absorbance (A_r) was calculated as follows:

$$A_r = \left(\frac{A_1 + A_2}{2} - A_c \right) \times K$$

where A_1 and A_2 denote the absorbance of duplicate wells, A_c the absorbance of the same sample obtained for the control well, and K the inverted value of the mean of the differences between the absorbance with the anti-HSA and the control antigen obtained for RF-ref in dilution 1/800. For each plate, correction with K gives an A_r value of 1.0 for the RF-ref diluted 1/800.

Reference titration curves for IgG-RF, IgA-RF and IgM-RF were drawn with the A_r values of the RF-ref on the ordinate and dilutions of the RF-ref in geometrical progression on the abscissa (Fig. 2). Each absorbance value in the curves is the mean of four different measurements. For each sample, the dilution of RF-ref which gave the same A_r as the test sample was read off from the reference curve (Fig. 2). The ratio of this dilution and the dilution of the sample was calculated and multiplied by 1,000, since the RF-ref was said to contain 1,000 AU/ml of IgG-RF, IgA-RF and IgM-RF.

Other reagents and laboratory variables

Affinity purified RF. RA serum heated at 56 °C for 30 min and absorbed with E was further absorbed on a rabbit IgG-Sepharose column. After washing with VBS the column was eluted with 4 mol/l $MgCl_2$. The eluate was dialysed against 0.15 mol/l NaCl and concentrated to the original volume by ultrafiltration (7).

C1q was purified by affinity chromatography essentially as

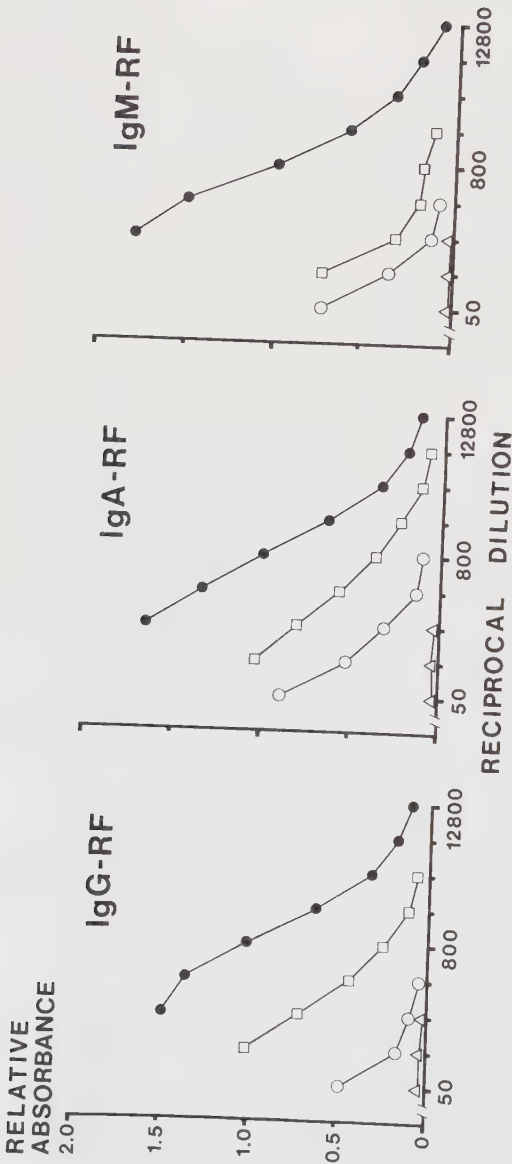


Fig. 2. Titration curves for IgG-RF, IgA-RF and IgM-RF using local RF-reference serum (●), the WHO RF reference preparation, 50 IU/ml (○), RF positive patient serum (□) and RF negative blood donor serum (△).

described by McKay (16).

C4 and C1r-C1s-C1IA complexes were measured by electro-immunoassay (17, 18).

Circulating immune complexes (CIC). C1q binding substances were measured by the C1q binding assay (19) and by the C1q deviation test (20). The results were expressed as equivalents of heat aggregated IgG in mg/l of serum (8).

IgG, IgA and IgM were measured by electroimmunoassay (21, 22).

Waller-Rose test was performed according to standard procedures (23).

Patients

Sera from a group of RA patients, previously described (8) were studied. The group comprised 16 patients (3 men, 13 women) with active classical rheumatoid arthritis (ARA), aged 33-70 (mean age 55) years. All patients were or had been Waller-Rose test positive. Podophyllotoxin derivatives (PTD) were given in daily doses of between 200 and 300 mg. Treatment was interrupted after about 6 months (5-7 months). The patients were examined at the start of treatment, at the end of therapy, and 6 weeks after withdrawal of the drug.

As an expression for the activity of joint inflammation, a synovitis index was evolved by adding 2 points for each markedly swollen joint and 1 point for each slightly swollen one.

Samples from other patients with classical rheumatoid arthritis (ARA) were used in some experiments.

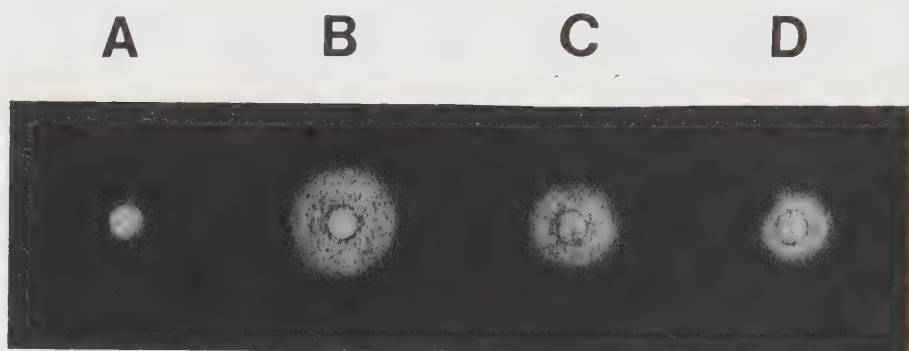


Fig. 3. Haemolysis in gel assay for C activating RF. (A), Normal human serum heated at 56 °C for 30 min and absorbed with sheep erythrocytes, (B), RA serum heated at 56 °C for 30 min and absorbed with sheep erythrocytes, (C), RA serum as in B but also absorbed with rabbit IgG-Sepharose and (D), RF-containing eluate from IgG-Sepharose.

Statistics

Reproducibility was expressed as the standard deviation in per cent of the mean. Spearman rank correlation test was used for analysing correlation, and the Wilcoxon matched-pairs signed-rank test for calculating the significance of differences (24).

RESULTS

Measurement of C activating RF

In the HIG assay for C activating RF, all patient sera gave lytic zones with EAGr, zones of full lysis being generally obtained (Fig 3.B), though partial lysis was observed with a few sera. Virtually identical zones were obtained when paired serum and plasma samples were analysed. Dilutions of patient sera gave titration curves that were parallel to the curve obtained with RF-ref. (Fig. 1). The levels of C activating RF in 16 RA patients before treatment with PTD are shown in Fig. 4.

Absorbtion of RA serum on rabbit IgG-Sepharose resulted in a reduction of the size of the haemolytic zone (Fig. 3 C). The eluate from IgG-Sepharose contained C activating RF as shown in Fig. 3 D. Dilutions of this RF preparation gave a titration curve parallel to that obtained with the RF-ref (Fig. 1).

Reproducibility was estimated following 20 measurements with one serum in the low range and one serum in the high range on duplicate plates. The standard deviation was 16% in the low range and 14% in the high range.

Results obtained with 16 RA sera in the haemolytic tube assay (2), correlated closely with the lytic areas in the HIG assay for C activating factors ($r=0.89$) (Fig.5), and with inhibition areas in the HIG inhibition assay (7) ($r=0.84$). The zone areas in the two different HIG assays were also closely correlated ($r=0.81$).

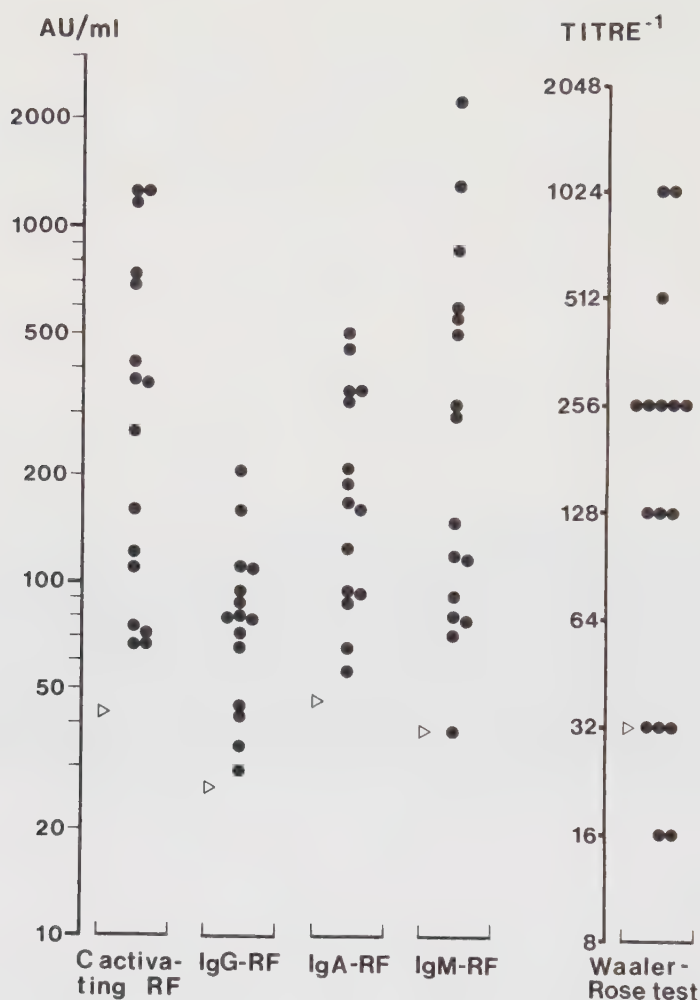


Fig. 4. Results of haemolysis in gel assay for C activating RF and the ELISA for class-specific RF in 16 patients with rheumatoid arthritis (IgG-RF and IgA-RF 15 patients) before treatment with podophyllotoxin derivatives. Values are given in arbitrary units (AU) per ml. For comparison Waaler-Rose test titres are shown. Values with the WHO RF reference preparation, stated to contain RF at 50 IU/ml, are indicated by triangles.

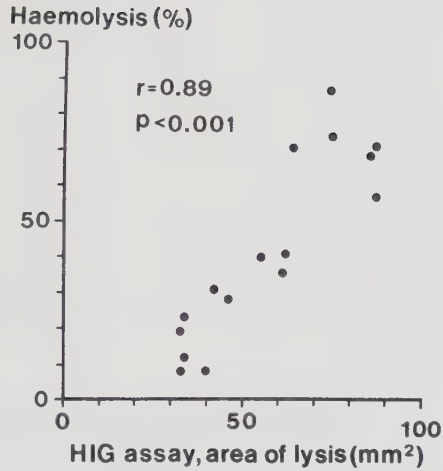


Fig. 5. Comparison of lytic areas obtained in the haemolysis in gel assay for C activating RF and per cent haemolysis in a haemolytic tube assay for RF. In both assays sheep erythrocytes are sensitized with reduced and alkylated rabbit IgG antibodies. Results with 16 rheumatoid arthritis sera are shown.

ELISA for IgG-RF, IgA-RF and IgM-RF

Blocking of residual protein binding capacity of the plastic surface was adequate, even at high antibody concentrations. With sera diluted 1/50, the absorbance values obtained with the control antigens were generally <0.2 . With different sera, titration curves (relative absorbance) were fairly parallel for both IgG-RF, IgA-RF and IgM-RF (Fig. 2). Addition of excess C1q, up to about 30% of its concentration in whole serum, to RF containing sera diluted 1/100 did not influence the estimates of RF in the ELISA. Comparable results were obtained for paired serum and plasma samples, which were tested in a dilution of 1/100 and, if necessary, in dilutions of up to 1/4,000. IgG-RF, IgA-RF and IgM-RF values in the 16 RA patients before PTD-treatment are shown in Fig. 4.

Throughout the study, duplicate wells differed only slightly in absorbance, in general less than 10% of the mean. Samples were reinvestigated if the difference exceeded 30%. Reproducibility was examined for IgG-RF at high (1.2) and at low (0.4) relative absorbance. Each sample was determined 20 times on separate plates and on different days. The standard deviation was 13% in the low range and 11% in the high range. As judged from duplicate determinations (data not shown), the reproducibility was similar for IgG-RF, IgA-RF and IgM-RF determinations.

With three samples from one patient, who was IgA deficient (IgA < 0.05 g/l), high absorbance values were constantly obtained with the control antigens in the assay for IgG-RF, and therefore IgG-RF from this patient was not reported. As might be expected, sera from this patient did not contain IgA-RF.

Correlations between C activating RF and class-specific RF

The concentrations of C activating RF, as measured by the HIG method, were correlated with those of IgM-RF ($r=0.86$), but not with the IgG-RF or IgA-RF values (Table 1). IgG-RF, IgA-RF and IgM-RF varied fairly independently of each other, the closest correlation being between IgA-RF and IgM-RF ($r=0.47$).

Relations between C activating RF and CIC

With the C1q deviation test, CIC were found in all sera but one (median 605, range $<10 - 1850$), and in all sera but two with the C1q binding assay (median 650, range $<50 - 1725$). The occurrence of partial lysis in the HIG assay seen with some sera was unrelated to high concentrations of CIC. The amounts of C activating RF showed a marked correlation with the concentrations of CIC as measured by the C1q binding assay ($r=0.88$), but not as measured by the C1q deviation test ($r=0.32$) (Table 1).

C1 activation and C activating RF

Measurements of $C1r-C1s-C1IA$ complexes by electroimmunoassay gave high values, in the range between 25 and 56 % of the reference (normal range 11 - 25 %). The concentrations of $C1r-C1s-C1IA$ complexes were not significantly correlated with those of C activating RF ($r=0.38$) (Table 1). The concentrations of C4 showed a negative but insignificant correlation with the C activating RF, ($r=-0.22$).

C activating RF and disease activity

Disease activity, as reflected by the synovitis index, was not correlated with the C activating RF or with any of the class-specific RF (Table 1). After administration of PTD for 6 months reduced concentrations of RF, as measured with the different

Table 1. Correlation coefficients (Spearman rank correlation) between C activating and class-specific rheumatoid factors and 5 other variables studied in 16 patients with rheumatoid arthritis before treatment with podophylotoxin derivatives.

C activating RF	IgG-RF	IgA-RF	IgM-RF	C4	C $\bar{1}$ r-C $\bar{1}$ s- C $\bar{1}$ IA	C1qDV	C1qBA
IgG-RF ¹	0.22						
IgA-RF ¹	0.43	0.00					
IgM-RF	0.86***	0.34	0.47				
C4	-0.22	-0.01	-0.03	-0.01			
C $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA ^a	0.38	0.27	0.25	0.13	-0.50		
C1qDV ^b	0.32	0.16	0.37	0.27	-0.20*	0.64*	
C1qBA ^c	0.88***	0.32	0.37	0.72**	-0.42	0.24	0.14
SF ^d	0.12	-0.06	0.35	0.03	-0.51**	0.66*	0.00

^aC $\bar{1}$ r-C $\bar{1}$ s-C $\bar{1}$ IA complexes.

^bCirculating immune complexes in C1q deviation test.

^cCirculating immune complexes in C1q binding assay.

^dSynovitis index. A patient with fibrosing alveolitis was excluded.

¹One IgA deficient patient was excluded.

* $p \leq 0.05$ (two-tailed test)

** $p < 0.01$

*** $p < 0.001$

assays, were noted ($p < 0.001$) (Table 2). The relative reduction of class-specific RF was more pronounced than that of each corresponding immunoglobulin class ($p < 0.01$ for IgA and IgM and $p < 0.05$ for IgG). After withdrawal of PTD, RF in serum approached the values seen before the start of treatment.

DISCUSSION

A simple HIG assay, useful for direct measurement of C activating RF in clinical samples was described. In a previous study (7), RF were shown to inhibit immune haemolysis of cells coated with intact rabbit IgG antibodies in a similar assay system. The use of reduced and alkylated IgG antibodies for sensitization of the cells provided a means of demonstrating immune haemolysis mediated by RF. The close correlation ($r = 0.81$) between the two HIG assays suggests that the same population of RF molecules was responsible - i.e., in the previous assay the presence of RF inhibited immune haemolysis, but in the assay described here C activation by the RF is a prerequisite for the lytic reaction. RF without C activating properties, such as IgA-RF, were probably of limited consequence in the assays. Accordingly, serum from an IgA deficient patient with RA contained C activating RF and also inhibited lysis of cells coated with intact IgG. Factors other than RF, such as immune complexes, may inhibit immune haemolysis (7), and for this reason the HIG test in which RF mediate immune haemolysis should be the more specific assay for C activating RF.

Although the use of undiluted sera in the HIG test might result in the interference of other serum factors with RF mediated immune haemolysis, the similar results obtained with the haemolytic tube assay using more diluted samples (2) and the HIG assay (Fig. 5) argue against this having happened.

Table 2. Changes during treatment with podophyllotoxin derivatives (PTD) of serum rheumatoid factors and immunoglobulin levels (16 patients).

	Start of treatment		End of treatment (6 months)		Off drugs (6 weeks)	
	Median	(range)	Median	Change from start range	Median	Change from end range p
C activating RF (AU/ml ¹)	310	(67-1250)	98	-18/-1901	179 ¹	+928/-1 <0.001
IgG-RF (AU/ml)	79 ¹	(29-202)	34 ¹	+15/-87	64 ²	+142/-13 <0.01
IgG (g/l ^b)	18	(10-25)	13	0/-11	15 ¹	+6/-2 <0.01
IgA-RF (AU/ml)	167 ¹	(56-500)	61 ¹	-38/-343	97 ²	+173/-49 <0.05
IgA (g/l ^c)	2.7 ¹	(1.5-4.6)	2.1 ¹	+0.6/-1.9	2.3 ²	+1.6/-0.8 n.s. ³
IgM-RF (AU/ml)	221	(38-2026)	58	-24/-1618	154 ¹	+1725/-18 <0.01
IgM (g/l ^d)	1.3	(0.4-1.9)	0.8	0/-0.9	0.9 ²	+0.9/-0.4 <0.05

^aRheumatoid factors are expressed in arbitrary units (AU) referring to a reference serum said to contain 1,000 AU/ml

^bReference area 6.5 - 15 g/l

^cReference area 0.7 - 2.4 g/l

^dReference area 0.3 - 1.2 g/l

¹15 patients

²14 patients

³n.s. = not significant

Furthermore, an affinity purified RF preparation gave a dose-response curve parallel to curves obtained with RF containing sera (Fig. 1). Although the size of the lytic zones did not appear to be affected, it seems quite likely that the partial lysis occasionally seen was caused by interfering substances.

An ELISA was employed in order to relate C activating RF with class-specific RF. Several test procedures have been described that are based on the principle of binding RF to insolubilized IgG, followed by a visualization procedure with indicator antibodies specific for the various immunoglobulin classes (25-27). As pointed out by Tarkowski et al., (28), the interaction between RF and Fc fragments of indicator reagents will cause false-positive results in such assays, unless conjugated $F(ab')_2$ or F(ab) fragments are used. Another possible error in RF assays with rabbit immunoglobulin as antigen is the presence of antibodies to rabbit IgM frequently to be found in IgA deficiency (29) but not in RA (30). For this reason a rabbit IgG fraction was used for coating the microtitre plates. To our knowledge, antibodies reactive with rabbit IgG, other than RF, have not been reported in human sera. As shown by the titration curves, blocking of binding sites for RF of one immunoglobulin class with RF of another class was not seen with the conditions used. Furthermore, the possible competition between RF and C1q for binding sites in the Fc part of the IgG anti-HSA molecule was negligible.

With serum samples from an IgA deficient patient with RA, high absorbance values, were obtained with the control antigens alone (i.e. HSA and gelatin) when anti- γ conjugates were used but not when anti- μ conjugates were used. The reason for this is not clear. The presence of IgG antibodies to HSA might be assumed since such antibodies have been found in some human sera (31,32).

In the RA patients studied, IgM class RF apparently predomi-

nated as activators of C mediated immune haemolysis. This is in accordance with the findings of Sabharwal et al., (33) who showed that RF in serum produced C activation similar to that produced by purified IgM-RF, and that (on a weight basis) IgM-RF are up to 1,000 times as effective than IgG-RF in their ability to activate C. In partial contrast to our findings, Kaplan et al., (34) reported that RF determined by a haemolytic assay (2) correlated somewhat more closely to IgG-RF than to IgM-RF. However, their RA patients differed from ours by showing more pronounced extra-articular manifestations and lower C4 levels. RA patients with complicating systemic vasculitis frequently have high concentrations of IgG-RF and manifest hypocomplementaemia (35).

The concentrations of C activating RF, and IgM-RF were closely correlated with those of CIC obtained by the C1q binding assay. At least, this suggests that in RF containing sera, IgM-RF is an important constituent of the C1q binding material detected with this well established immune complex assay.

In a previous study (8), we found that the concentrations of C1r-C1s-C1IA complexes in serum from RA patients, indicating C1 activation, were related to the extent of synovial inflammation as expressed by a synovitis index. With the present findings, bearing in mind that the assays used might not detect complexed RF, C1 activation could not be fully accounted for by increased RF concentrations. It is not excluded that C1r-C1s-C1IA complexes in serum partly derive from inflamed tissues. Apart from RF, antibodies to collagen (36), CRP containing complexes (8) and granulocyte proteases (37) might be considered as possible C1 activators in RA.

Administration of PTD clearly reduced the serum concentrations of RF. Immunoglobulin concentrations were decreased to a much lesser extent than those of IgG-RF, IgA-RF and IgM-RF. Treatment

with penicillamine has been reported to result in a selective decrease of IgM-RF as compared with IgM, though no corresponding relationship was found between IgG-RF and serum IgG (38). The apparent effect of these clinically efficient drugs on RF synthesis lends support to a possible pathogenic role of RF in RA.

ACKNOWLEDGEMENT

This work was supported by grants from the Swedish Medical Research Council (B83-16X-68-19C), the Medical Faculty University of Lund, the Thorsten and Elsa Segerfalks Foundation for Medical Research and Education, Forssmans fond, Föreningen för bistånd åt Vanföra i Skåne, Greta och Johan Kocks Stiftelser, Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism and Alfred Österlunds Stiftelse.

REFERENCES

- 1 Johnson P M, Faulk W P. Rheumatoid factor: its nature, specificity and production in rheumatoid arthritis. Clin Immunol Immunopathol 1976; 6: 414-30.
- 2 Tanimoto K, Cooper N R, Johnsson J S, Vaughan J H. Complement fixation by rheumatoid factor. J Clin Invest 1975; 55: 437-45.
- 3 Taylor-Upsahl M M, Johnson P M, Mellbye O J, Natvig J B. A study of complement fixation by rheumatoid factor using a haemolytic assay system. Clin exp Immunol 1977; 28: 204-11.
- 4 Ziff, M. Pathophysiology of rheumatoid arthritis. Fed Proc 1973; 32: 131-3.
- 5 Vaughan J H. Rheumatoid arthritis, rheumatoid factor and the Epstein-Barr virus. J Rheum 1979; 6: 381-8.
- 6 Scott D G I, Bacon P A, Allen C, Elson C J, Wallington T. IgG rheumatoid factor, complement and immune complexes in rheumatoid synovitis and vasculitis: comparative and serial studies during cytotoxic therapy. Clin exp Immunol 1981; 43: 54-63.
- 7 Truedsson L, Sjöholm A G. Inhibition of complement-mediated hemolysis in gel by rheumatoid factors. Acta path microbiol immunol scand Sect C, in press.

- 8 Berglund K, Laurell A-B, Nived O, Sjöholm A G, Sturfelt G. 111
Complement activation, circulating C1q binding sub-
stances and inflammatory activity in rheumatoid arthritis:
relations and changes on suppression of inflammation. J
Clin Lab Immunol 1980; 4: 7-14.
- 9 Anderson S G, Bentzon M W, Houba V, Krag P. Interna-
tional reference preparation of rheumatoid arthritis serum.
Bull Wld Hlth Org 1970; 42: 311-8.
- 10 Johnson B A, Hoffmann L G. Effect of reduction and al-
kylation on structure and function of rabbit IgG antibody -
I. Effect on ability to activate complement depends on
conditions of reduction. Mol Immunol 1981; 18: 181-8.
- 11 Rapp H J, Borsos T. Molecular basis of complement action.
New York: Appleton-Century-Crofts, 1970.
- 12 Truedsson L, Sjöholm A G, Laurell A-B. Screening for
deficiencies in the classical and alternative pathways of
complement by hemolysis in gel. Acta path microbiol scand
Sect C 1981; 89: 161-6.
- 13 Stanworth D R, Turner M W. Immunochemical analysis of
immunoglobulins and their sub-units. In: Weir DM, ed.
Handbook of experimental immunology. 3 ed. Oxford, London,
Edinburgh, Melbourne: Blackwell Scientific Publications,
1978: 6.19-6.22.
- 14 Laemmli U K. Cleavage of structural proteins during the
assembly of the head of Bacteriophage T4. Nature 1970; 227:
680-5.

- 15 Voller A, Bidwell D E, Bartlett A. Enzyme immunoassays in diagnostic medicine. Bull Wld Hlth Org 1976; 53: 55-65.
- 16 McKay EJ. A simple two-step procedure for the purification of plasma C1q from different animal species. Immunol Lett 1981; 3: 303-8.
- 17 Sjöholm A G. Complement components in normal serum and plasma quantitated by electroimmunoassay. Scand J Immunol 1975; 4: 25-30.
- 18 Laurell A-B, Mårtensson U, Sjöholm A G. Quantitation of $\bar{C}1r$ - $\bar{C}1s$ - $\bar{C}1$ inactivator complexes by electroimmunoassay. Acta path microbiol scand Sect C 1979; 87: 79-81.
- 19 Zubler R H, Lange G, Lambert P H, Miescher P A. Detection of immune complexes in unheated sera by a modified ^{125}I C1q-binding test. Effect of heating on the binding of C1q by immune complexes and application of the test to systemic lupus erythematosus. J Immunol 1976; 116: 232-5.
- 20 Sobel A T, Bokisch V A, Müller-Eberhard H J. C1q deviation test for the detection of immune complexes, aggregates of IgG, and bacterial products in human serum. J Exp Med 1975; 142: 139-50.
- 21 Laurell C-B. Electroimmunoassay. Scand J Clin Lab Invest 1972; 29, suppl. 124: 21-37.
- 22 Grubb O A. Crossed immunoelectrophoresis and electroimmunoassay of IgM. J Immunol 1974; 112: 1420-5.
- 23 Ball J. Serum factor in rheumatoid arthritis agglutinating

- 24 Siegel S. Nonparametric statistics for the behavioral sciences. New York: McGraw-Hill, 1956.
- 25 Hay F C, Nineham L J, Roitt I M. Routine assay for detection of IgG and IgM antiglobulins in seronegative and seropositive rheumatoid arthritis. Brit Med J 1975; 3: 203-4.
- 26 Gripenberg M, Wafin F, Isomäki H, Linder E. A simple enzyme immunoassay for the demonstration of rheumatoid factor. J Immunol Methods 1979; 31: 109-18.
- 27 March R E, Reeback J S, Holborow E J, Coombs R R A. MrsPAH: A simple microtitre plate test for rheumatoid factors of different classes. J Immunol Methods 1981; 42: 137-46.
- 28 Tarkowski A, Bjursten L M, Nilsson L-Å, Nygren H. False positive results in class-specific rheumatoid factor (RF) assays due to interaction between RF and Fc fragments of anti-immunoglobulin indicator reagents. J Immunol Methods 1983; 58: 171-82.
- 29 Truedsson L, Axelsson U, Laurell A-B. Frequent occurrence of anti-rabbit IgM in IgA deficiency. Acta path microbiol scand Sect C 1982; 90: 315-20.
- 30 Truedsson L, Sturfelt G. False positive Waaler-Rose test due to antibodies to rabbit IgM in IgA deficiency. Clin Exp Rheum, in press.
- 31 Lindström P, Wager O. IgG autoantibody to human serum albumin

studied by the ELISA-technique. Scand J Immunol 1978; 7: 419-25.

- 32 Onica D, Calugaru A, Margineanu I, Zamfir G, Belascu I.
Immunochemichal characterization of anti-albumin antibodies
in liver diseases. Clin Immunol Immunopath 1983; 26: 223-31.
- 33 Sabharwal U K, Vaughan J H, Fong S, Bennett P H, Carson D A,
Curd J G. Activation of the classical pathway of complement
by rheumatoid factors: assessment by radioimmunoassay for C4.
Arthritis Rheum 1982; 25: 161-7.
- 34 Kaplan R A, Curd J G, Deheer D H, Carson D A, Pangburn M K,
Müller-Eberhard H J, Vaughan J H. Metabolism of C4 and factor
B in rheumatoid arthritis. Arthritis Rheum 1980; 23:911-20.
- 35 Elson C J, Scott D G I, Blake D R, Bacon P A, Holt P D J.
Complement-activating rheumatoid-factor-containing complexes
in patients with rheumatoid vasculitis. Ann Rheum Dis 1983;
42: 147-50.
- 36 Trentham D E. Collagen arthritis as a relevant model for
rheumatoid arthritis: evidence pro and con. Arthritis Rheum
1982; 25: 911-6.
- 37 Hadler N M, Spitznagel J K, Quinet R J. Lysosomal enzymes in
inflammatory synovial effusions. J Immunol 1979; 123: 572-7.
- 38 Wernick R, Merryman P, Jaffe I, Ziff M. IgG and IgM rheumatoid
factors in rheumatoid arthritis: quantitative response to
penicillamine therapy and relationship to disease activity.
Arthritis Rheum 1983; 26: 593-8.

JIM 02761

Human Factor D of the Alternative Pathway: Purification and Quantitation by Enzyme Amplified Electroimmunoassay

Lennart Truedsson ^{1,*} and Gunnar Sturfelt ²

¹ *Department of Immunology, Institute of Medical Microbiology, University of Lund, and* ² *Department of Rheumatology, University Hospital of Lund, Lund, Sweden*

(Received 22 November 1982, accepted 21 April 1983)

Purified factor D was prepared with a yield of about 30%. Monospecific antiserum was raised in rabbits. Immunochemical quantitation of factor D in serum and plasma was performed by electroimmunoassay and a sensitive staining technique based on enzyme amplification. Peroxidase-labeled swine antibodies to rabbit immunoglobulin were applied to the gel after electrophoresis. Immune precipitates were then visualized by staining for peroxidase activity. Factor D could be detected at 50 µg/l. The normal concentration of D in serum was 1.6 mg/l, as assessed by this assay.

Key words: *complement — factor D — factor D purification — electroimmunoassay — immunoenzyme technique*

Introduction

Factor D (D) is a plasma protein playing an essential part in activation of the alternative complement (C) pathway (Müller-Eberhard and Götze, 1972; Hunsicker et al., 1973). It is a serine protease (Brade et al., 1974; Fearon et al., 1974) catalyzing cleavage of factor B (B) in the presence of C3b and Mg²⁺ (Lesavre et al., 1979), which results in the generation of the C3b, Bb complex with C3 cleaving activity.

Because of the low serum concentration of D, 1–2 mg/l (Lesavre and Müller-Eberhard, 1978; Lesavre et al., 1979), quantitation by ordinary immunochemical methods based on precipitation in gel is not possible. Functional assays have been described which use hemolysis of guinea pig erythrocytes (Martin et al., 1976), C3b-coated sheep erythrocytes (Lesavre and Müller-Eberhard, 1978) or rabbit erythrocytes (Lesavre et al., 1979). Measurement of D by radioimmunoassay has also been described (Lesavre and Müller-Eberhard, 1978).

* Address correspondence to: Dr. L. Truedsson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Löfberg and Grubb (1979) reported that, by introducing an enzyme amplifying technique, it was possible to increase the sensitivity of single radial immunodiffusion. In this study, we have found that this amplifying technique applied to electroimmunoassay results in an immunochemical method sensitive enough to be used in the determination of D in serum and plasma.

Materials and Methods

Normal serum and plasma

Blood specimens with 5 mmol/l EDTA, and samples without anticoagulant, were obtained from healthy blood donors. After centrifugation, serum and plasma were frozen at -80°C within 5 h of collection.

Reference serum

Sera from 148 normal blood donors were pooled and stored in aliquots at -80°C . This reference serum pool contained 100% D.

D-depleted serum (RD)

RD was obtained by gel filtration of fresh human serum on Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala) (Martin et al., 1976), or by passing fresh serum over a column of Bio-Rex 70 (Bio-Rad. Lab., Richmond, CA), as described by Lesavre et al. (1979).

Purification of D

D was purified from frozen human ACD plasma by a combination of ion-exchange chromatography and gel filtration as outlined by Volanakis et al. (1977). The fractions were tested for D by hemolytic assay, as originally described by Martin et al. (1976). Ultrafiltration on Amicon PM 10 membranes (Amicon Corp., Lexington, MA) was used to concentrate pooled D-containing fractions. The conductance of the buffers refers to values at 20°C .

Purification consisted of the following steps:

(1) After addition of 2 mmol/l EDTA, 3.5 l plasma was adjusted to pH 7.3 with 1 mol/l HCl and to a conductance of 11.0 mS/cm by dilution with distilled water. The plasma was then applied to a column of Bio-Rex 70 (7 cm $\times$ 23 cm) equilibrated with 50 mmol/l sodium phosphate, 2 mmol/l EDTA, pH 7.3 with a conductance adjusted to 11.0 mS/cm with NaCl (PBS). The column was washed, first with 1.5 l PBS containing 0.2% Triton X-100 (Eastman Kodak, Rochester, NY) (Lesavre et al., 1979) and then with 4.5 l PBS. The bound material was eluted with PBS adjusted to 30 mS/cm with a flow rate of 200 ml/h. As described by Tenner et al. (1981) D and Clq appeared in the same fractions under these conditions.

(2) The pooled fractions containing D (300 ml) were concentrated to 20 ml and applied to a column (4.5 cm $\times$ 90 cm) of Sepharose Cl-6B (Pharmacia Fine Chemicals, Uppsala) equilibrated with PBS, 40 mS/cm. The flow rate was 120 ml/h. The use of this gel made it possible to purify both D and Clq simultaneously.

(3) The D pool (400 ml), concentrated to 7.5 ml, was centrifuged to remove visible precipitates and the supernatant was filtered on a column (2.6 cm $\times$ 100 cm) of AcA 44 (LKB-Produkter AB, Bromma) equilibrated with 0.1 mol/l Tris (Sigma Chemicals, St. Louis, MO), 0.4 mol/l NaCl, 2 mmol/l EDTA, pH 7.5. The flow rate was 20 ml/h.

(4) The fractions containing D (95 ml) were dialyzed against 10 mmol/l barbital buffer, pH 7.4, adjusted to 15 mS/cm with NaCl (VBS). The dialysate was applied to a column of Bio-Rex 70 (2.3 cm $\times$ 6 cm) equilibrated with VBS and washed with 120 ml of this buffer. The bound material was eluted with the barbital buffer adjusted to 35 mS/cm. The flow rate was 60 ml/h, and 4 ml fractions were collected. Eight fractions with the highest D activity, comprising one-third of the fractions containing D, were pooled and concentrated to 2.6 ml. This preparation had a protein content of 0.55 g/l (or a total of 1.43 mg), as measured by the method of Lowry et al. (1951), with bovine serum albumin as reference protein.

Production of antiserum

The D preparation, 0.5 ml, was emulsified with 0.5 ml complete Freund's adjuvant (Difco Lab., Detroit, MI). The emulsion was injected subcutaneously at several points on the back of a rabbit. A booster injection was given 3 weeks later, and blood was collected after a further week, and every 2 weeks thereafter.

Other reagents

Rabbit anti-C5 antiserum was available in the laboratory.

Purified Clq was prepared by affinity chromatography as described by McKay (1981).

Enzyme amplified electroimmunoassay (EAE)

Electroimmunoassay was according to Laurell (1972), using 75 mmol/l barbital buffer containing either 2 mmol/l EDTA (VB-EDTA) or 2 mmol/l Ca^{2+} in the gel. After preliminary trials with agaroses having different endosmotic characteristics, a low endosmosis agarose, Agarose (LE) (FMC Corp., Marine Colloids Div., Rockland, ME) 1% (w/v) was found to be the most suitable. The gel was moulded on glass plates or preferably on agarose-coated polyester films (Gel Bond film; FMC Corp., Marine Colloids Div., Rockland, ME). To obtain distinct immunoprecipitates, 5% (w/v) polyethylene glycol (PEG) 6000 (Kebo AB, Stockholm) was added to the gel. Addition of PEG 20000 (Kebo AB, Stockholm) or Dextran T10 (Pharmacia Fine Chemicals, Uppsala) failed to improve the precipitation peaks. Antiserum to D, at a concentration of 0.07% (v/v), was added to the agarose. Five microliters of the samples in dilution 1/2 in VB-EDTA were introduced into 3 mm diameter wells in the agarose. Electrophoresis was carried out for 5 h at 20 V/cm. The gel was washed in 0.15 mol/l NaCl for 30 min, covered with a filter paper moistened with saline and a thick wad of soft absorbent paper on top, and pressed for about 10 min. The washing procedure was repeated twice.

The amplification technique described by Löfberg and Grubb (1979) for enzyme amplified single radial immunodiffusion was used to visualize the immunoprecipi-

tates. After washing, the gel was flooded with horseradish peroxidase-conjugated swine antibodies against rabbit immunoglobulins (Dakopatts, Copenhagen), diluted 1/20 in 50 mol/l phosphate buffered saline, pH 7.4, to which 5% non-immune swine serum was added. The gel was incubated with the labeled antibody in a moist chamber for 30 min. The washing procedure was then repeated 3 times. The immunoprecipitates were stained wine-red within 10–15 min on being immersed in a bath of 20 mg 3-amino-9-ethylcarbazole (Sigma Chemicals, St Louis, MO) dissolved in 2.5 ml dimethylformamide (BHH Chemicals, Poole), 50 ml 50 mmol/l acetate buffer, pH 5.0, and 25 μ l hydrogen peroxide 30% (v/v) (Graham et al., 1965). After rinsing in water the gel was air-dried.

Immunochemical and electrophoretic methods

Double immunodiffusion in gel (Ouchterlony, 1967) was performed in a gel of the same composition as that used in the EAE, and the precipitates were visualized by the enzyme amplifying technique.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970).

Hemolysis in gel (HIG) assay for D

The hemolytic diffusion plate assay described by Martin et al. (1976) was used with minor modifications. Agarose plates containing guinea pig erythrocytes were prepared as described previously (Truedsson et al., 1981) with the addition of 5% (v/v) RD reagent. The buffer used was veronal buffered saline containing 5 mmol/l Mg^{2+} and 10 mmol/l EGTA (VBS- Mg^{2+} -EGTA) (Truedsson et al., 1981). Wells, 3 mm in diameter, were punched and 5 μ l of the samples were introduced into each well. Incubation was carried out at 4°C for 16 h, and at 37°C for 2 h.

Results

Purified D

When analyzed by 12% SDS-PAGE the isolated protein gave only one band (Fig. 1), representing a molecular weight of approximately 25,000 daltons. When injected into a rabbit, the protein produced an antiserum which without absorption was monospecific for D. The plasma used for the preparation had a D concentration 78% of the reference serum (= 1.25 mg/l, see below) as determined by the EAE. Thus, the purification procedure resulted in 1.43 mg D from 3.5 l of the initial material, a yield of approximately 30%.

Antiserum specificity

Using unabsorbed anti-D antiserum, enzyme amplified double immunodiffusion gave rise to a single precipitation line, demonstrating a reaction of identity between D in normal human serum (NHS) and the purified D preparation (Fig. 2). Mixtures of equal volumes of the antiserum — heated at 56°C for 30 min — and NHS in serial dilutions, were tested by the HIG assay for D. Complete inhibition was

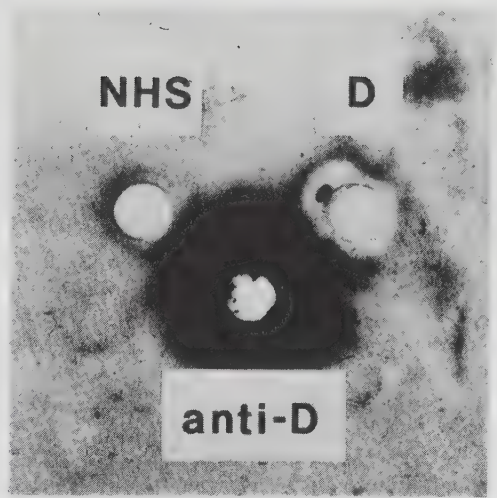


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the D preparation. Eleven micrograms of the preparation were applied to the gel, and the electrophoresis run under reducing conditions on a 12% gel. The anode was at the bottom.

Fig. 2. Enzyme amplified double immunodiffusion demonstrating an identity reaction between normal human serum (NHS) and purified D diluted 1:20 in VB-EDTA (D) obtained with anti-D antiserum diluted 1:4 in the same buffer (anti-D).

demonstrated with the anti-D antiserum in dilutions up to 1/16. Further dilution of the antiserum gave partial inhibition (Fig. 3A).

In a control experiment with antiserum to C5, another C component participating in the hemolytic reaction, the lytic pattern differed from that obtained with anti-D antiserum (Fig. 3B), indicating that inhibition by the anti-D antiserum is dependent upon specific binding of anti-D antibodies to D.

Enzyme amplified electroimmunoassay

Experiments with different buffers showed that in the presence of Ca^{2+} both

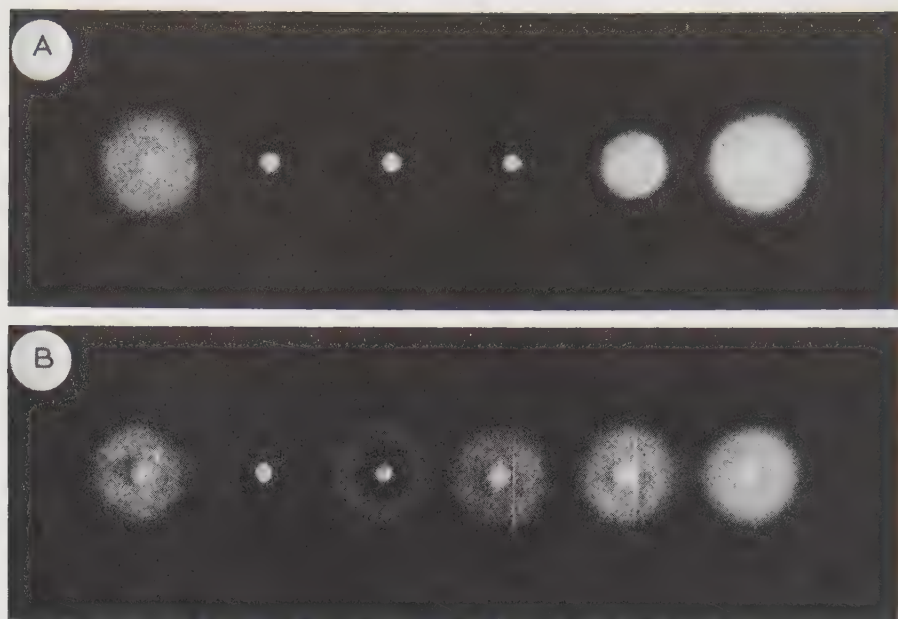


Fig. 3. A: influence of anti-D antiserum on D-activity in normal human serum (NHS) analyzed by the HIG assay for D. Reading from the left, the wells contain mixtures of: equal volumes of NHS and VBS-Mg²⁺-EGTA, NHS and anti-D antiserum undiluted and in dilutions 1:4, 1:16, 1:64 and 1:256. B: results, obtained from a control experiment with anti-C5 antiserum, performed in the same way.

anodal and cathodal precipitation peaks were obtained with NHS. With EDTA in the buffer only anodal peaks were observed (Fig. 4). VB-EDTA was therefore used throughout the study. No precipitation peaks were found with RD.

Purified D diluted in VB-EDTA gave more sharply pointed anodal peaks than NHS. When D was diluted in RD, the resultant peaks were the same in appearance as those seen with NHS. It was also noted that higher peaks were obtained with purified D diluted in RD than with corresponding dilutions of D in VB-EDTA.

In the absence of PEG 6000 the peaks became blurred, making the heights of the rockets difficult to estimate. Cathodally located stained material was occasionally noted with NHS (Fig. 4) and RD. Purified C1q gave rise to cathodal precipitates with similar appearance. This was also seen in the absence of antiserum in the gel.

Measurable precipitation peaks were obtained with the reference serum in dilutions up to 1/32 in VB-EDTA, thus permitting detection of D concentrations as low as 3% of normal. The precision of the EAE was estimated by 20 duplicate determinations made on different occasions with sera of varying D concentrations. The standard deviation was calculated to be 9.5% (Weeke, 1973).

D in normal serum and plasma

The D content of the purified preparation was estimated to be 354-fold that of the reference serum by EAE analysis of dilutions of the preparation in RD. This

value was the mean of 5 separate determinations. Thus, on the basis of a protein concentration of 0.55 g/l in the D preparation, the D concentration in the normal serum standard was calculated to be 1.6 mg/l.

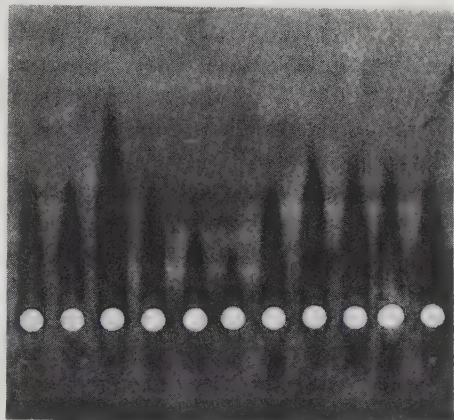


Fig. 4. Enzyme amplified electroimmunoassay of D in normal sera. From the left, precipitation peaks 3–6 represent the reference serum undiluted and in dilutions 1:2, 1:4 and 1:8.

D was quantitated in 20 normal human serum and plasma samples. The serum values varied between 65 and 140% (mean 102). Similar or somewhat lower values were obtained with corresponding plasma samples.

Discussion

The method of preparation resulted in highly purified D, as shown by analysis with SDS-PAGE. The yield was estimated as approximately 30%, which is higher than the values reported with previously described methods (Volanakis et al., 1977, Davis et al., 1979; Lesavre et al., 1979).

The introduction of the enzyme amplifying technique increases the sensitivity of the electroimmunoassay (Laurell, 1972) approximately 100-fold, and makes quantitation of D in serum possible. Low concentrations of D, approximately 50 μ g/l can be determined. The amplifying step also made it possible to use very low concentrations of antiserum in the gel, thus economizing with an expensive reagent. This modification of the electroimmunoassay is therefore suitable for determining serum proteins present in concentrations too low to be measured by ordinary immunochemical assays based on precipitation in gels.

By use of the EAE, the concentration of D in NHS was determined as 1.6 mg/l, which agrees with values reported by others (Lesavre and Müller-Eberhard, 1978; Lesavre et al., 1979).

It was possible to obtain distinct anodal precipitation peaks in the presence of

EDTA, by addition of PEG 6000 to the gel. The occasional appearance of cathodally located stained material was not caused by D-anti-D precipitates, since this phenomenon was seen also in the absence of antiserum.

It has been reported that under certain conditions Clq interacts with agarose gels resulting in the formation of precipitates (McKay, 1981) and when purified Clq was tested with the EAE, cathodally located stained precipitates were indeed obtained. Low dilutions of serum, as well as the presence of PEG 6000 in the gel, may favour such 'nonspecific' protein-agarose interaction. Binding of labeled swine antibodies to Clq is a possible mechanism for staining these precipitates.

The anodal and cathodal precipitation peaks obtained with NHS in the presence of Ca^{2+} confirms the results reported by Davis et al. (1979). They found that, after agarose gel electrophoresis, purified D, as well as D in NHS, were distributed on both the cathodal and anodal sides of the application site with the highest D-activity towards the anode. It was reported by Konno et al. (1978) that purified D has β -mobility, whereas D in NHS has α -mobility in the presence of EDTA. The binding of D to another serum protein in the absence of Ca^{2+} and Mg^{2+} has been proposed (Konno et al., 1978), and such complex formation might explain why precipitation peaks of purified D diluted in RD differed from those obtained with D diluted only in buffer.

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (B83-16X-68-19C), Greta och Johan Kocks Stiftelser, Konung Gustav V:s 80-årsfond, Riksförbundet mot reumatism and Alfred Österlunds Stiftelse.

References

- Brade, V., A. Nicholson, D. Bitter-Suermann and U. Hadding, 1974, *J. Immunol.* 113, 1735.
- Davis III, A.E., C. Zalusky, F.S. Rosen and C.A. Alper, 1979, *Biochemistry* 18, 5082.
- Fearon, D.T., K.F. Austen and S. Ruddy, 1974, *J. Exp. Med.* 139, 355.
- Graham, Jr., R.C., U. Lundholm and M.J. Karnovsky, 1965, *J. Histochem. Cytochem.* 13, 150.
- Hunsicker, L.G., S. Ruddy and K.F. Austen, 1973, *J. Immunol.* 110, 128.
- Konno, T., Y. Katsuno and H. Hirai, 1978, *J. Immunol. Methods* 21, 325.
- Laemmli, U.K., 1970, *Nature (London)* 227, 680.
- Laurell, C.-B., 1972, *Scand. J. Clin. Lab. Invest.* 29 (Suppl. 124), 21.
- Lesavre, P.H. and H.J. Müller-Eberhard, 1978, *J. Exp. Med.* 148, 1498.
- Lesavre, P.H., T.E. Hugli, A.F. Esser and H.J. Müller-Eberhard, 1979, *J. Immunol.* 123, 529.
- Löfberg, H. and A.O. Grubb, 1979, *Scand. J. Clin. Lab. Invest.* 39, 619.
- Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, *J. Biol. Chem.* 193, 265.
- McKay, E.J., 1981, *Immunol. Lett.* 3, 303.
- Martin, A., P.J. Lachmann, L. Halbwachs and M.J. Hobart, 1976, *Immunochemistry* 13, 317.
- Müller-Eberhard, H.J. and O. Götz, 1972, *J. Exp. Med.* 135, 1003.
- Ouchterlony, Ö., 1967, in: *Handbook of Experimental Immunology*, ed. D.M. Weir (Blackwell Scientific Publications, Oxford) p. 655.
- Tenner, A.J., P.H. Lesavre and N.R. Cooper, 1981, *J. Immunol.* 127, 648.
- Truedsson, L., A.G. Sjöholm and A.-B. Laurell, 1981, *Acta Pathol. Microbiol. Scand. Sect. C* 89, 161.
- Volanakis, J.E., R.E. Schrohenloher and R.M. Stroud, 1977, *J. Immunol.* 119, 337.
- Weeke, B., 1973, *Scand. J. Immunol.* 2 (Suppl. 1), 37.

COMPLEMENT FACTOR D CONCENTRATIONS IN NORMAL SERA: COMPARISON OF IMMUNOCHEMICAL AND FUNCTIONAL DETERMINATIONS

¹G. STURFELT and ²L. TRUEDSSON

¹Department of Rheumatology and ²Department of Immunology, Institute of Medical Microbiology,
University of Lund, Sweden

Sturfelt, G. & Truedsson, L.: Complement factor D concentrations in normal sera: comparison of immunochemical and functional determinations. *Acta path. microbiol. immunol. scand. Sect. C, 91: 383-389, 1983.*

The concentration of factor D (D) was determined in the sera of 144 healthy adults and 29 healthy children by enzyme amplified electroimmunoassay (EAE), and by a hemolysis in gel (HIG) assay for D. The 95% geometric confidence areas for D in adults were 75-143% as determined by EAE, and 65-171% by the functional assay. The concentrations were given in per cent of a reference serum pool containing 1.6 mg/l of D as measured by EAE. The correlation coefficient between D values obtained by the two methods was $r = 0.52$ ($p < 0.001$). D levels were lower in children than in adults. In the EAE purified D, and pathological sera with very high D concentrations, gave lower precipitation peaks with dilutions performed in buffer than with dilutions in D depleted serum or human serum albumin.

Key words: Complement; factor D; electroimmunoassay; immunoenzyme techniques; hemolytic assays.

G. Sturfelt, Department of Rheumatology, University Hospital, S-221 85 Lund, Sweden.

Received 7.vi.83 Accepted 4.vii.83

Factor D (D) of the alternative complement pathway is a protease with a molecular weight of 23,500 (Fearon *et al.* 1974) present in serum at low concentrations, 1-2 mg/l (Lesavre & Müller-Eberhard 1978; Lesavre *et al.* 1979; Truedsson & Sturfelt 1983). Previously D has usually been determined by functional assays (Martin *et al.* 1976; Wilson *et al.* 1979). By employing a sensitive staining technique based on enzyme amplification, an adequately sensitive electroimmunoassay was developed (Truedsson & Sturfelt 1983). In the present study, D was measured by this enzyme amplified electroimmunoassay (EAE) in normal adults and in children. The results were compared with D values obtained using functional assays.

MATERIALS AND METHODS

Normal sera and plasma. Blood samples were collected from 144 registered blood donors, 112 men and 32 women, aged 19-61 (median 40 years), 6 healthy subjects aged 60-80 and from 29 healthy children aged 2-10

years (median 6 years). After centrifugation serum and EDTA-plasma were frozen in aliquots at -80 °C within 5 hours of collection.

Reference serum. A reference serum was obtained by pooling sera from 148 normal blood donors. D values were given in per cent of this reference serum containing D at 1.6 mg/l (Truedsson & Sturfelt 1983). The reference serum was stored in aliquots at -80 °C.

Purification of D. D was purified from human ACD plasma as described earlier (Truedsson & Sturfelt 1983).

Anti-D antiserum. A monospecific antiserum was obtained by immunizing rabbits with purified D emulsified in an equal volume of Freund's complete adjuvant (Difco Lab., Detroit, Mich., USA) (Truedsson & Sturfelt 1983).

Immune absorbent for D. The IgG fraction of anti-D antiserum was prepared by precipitation with 33% ammonium sulfate, followed by chromatography on DEAE cellulose (DE 32) (W. & R. Balston Ltd., Maidstone, England). The antibodies were coupled to cyanogenbromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at 10 mg protein/ml gel, as recommended by the manufacturer.

D depleted serum (RD). A serum reagent depleted of D (RD) was obtained by the method described by Lesavre *et al.* (1979) or in some experiments by passing fresh norm-

TABLE 1. Arithmetic Mean, Standard Deviation, Geometric Mean and 95 % Geometric Reference Area for D in Serum Are Given. The Values Are Expressed in per Cent of the Reference

	Arith. mean	Stand. dev.	Geom. mean	95 % geom. reference area
Healthy adults (19-61 years, n = 144)				
EAE	105	17	104	75-143
HIG	109	25	106	65-171
Healthy children (2-10 years, n = 29)				
EAE	73	9	73	56-95
HIG	70	19	67	41-112

al serum through a column with the D immune adsorbent.

The RD gave neither lysis in the hemolysis in gel (HIG) assay for D (Martin *et al.* 1976), nor a precipitate in the EAE (Truedsson & Sturfelt 1983). The RD gave full lysis in a HIG assay for the classical pathway, but no lysis in a HIG test for the alternative pathway (Truedsson

et al. 1981). Addition of highly purified D to RD at a concentration corresponding to that of D in normal serum, completely restored the alternative pathway function, as assessed by the HIG test.

Other reagents. Human serum albumin (HSA) was purchased from Sigma Chemicals, St. Louis, Mo., USA.

Enzyme amplified electroimmunoassay. A detailed

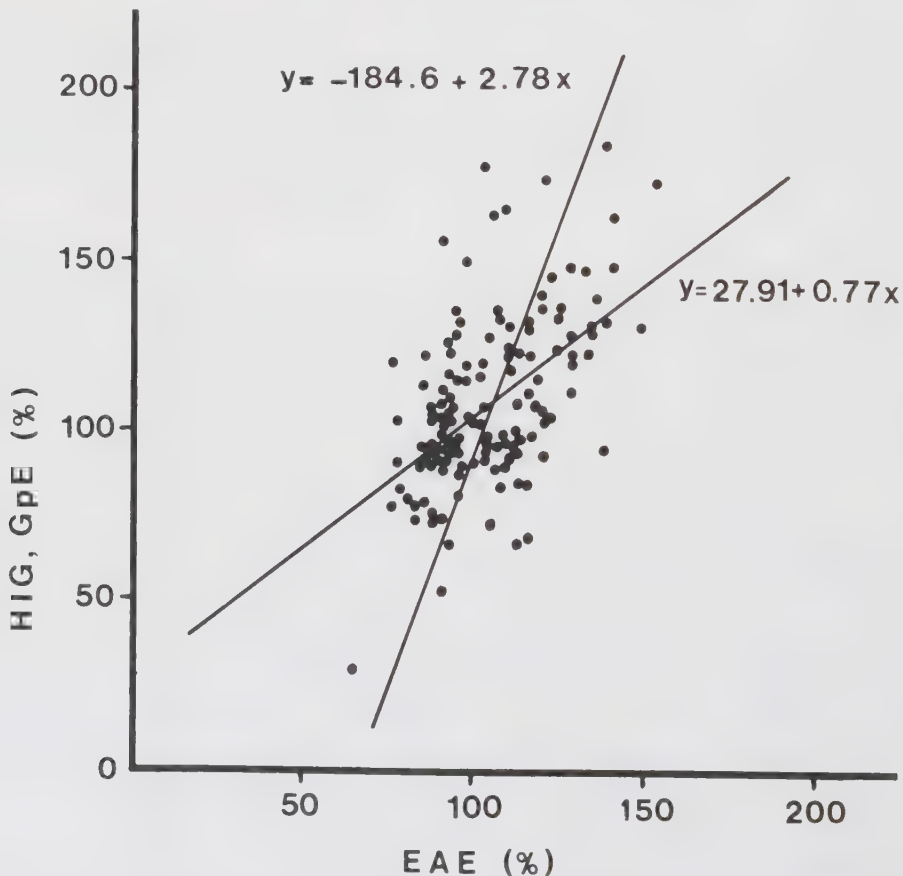


Fig. 1. D measured by EAE and HIG assay in sera from 144 normal subjects. Values are given in per cent of the reference.

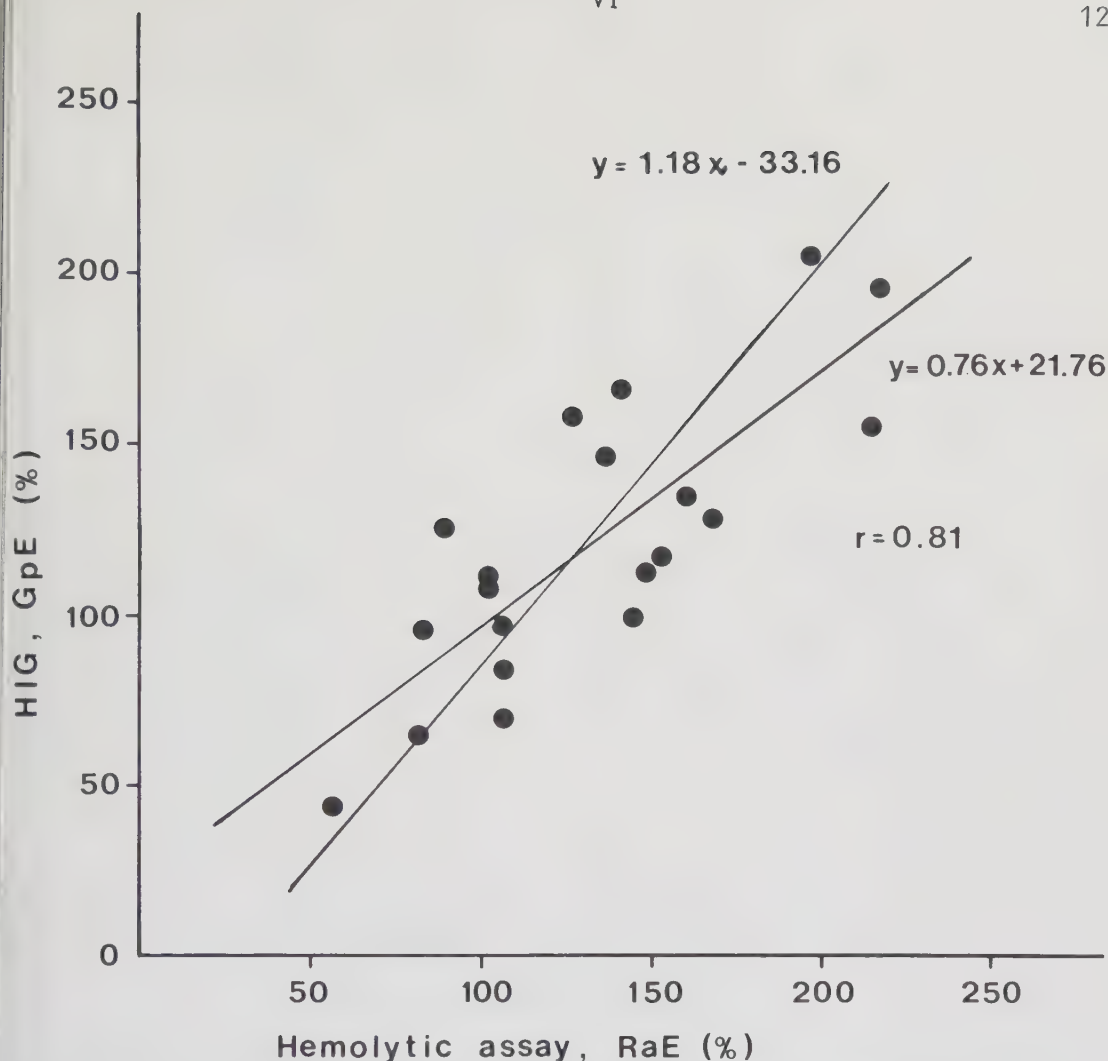


Fig. 2. Comparison of D activity measured by two hemolytic assays: HIG using guinea pig erythrocytes (HIG, GpE) and an assay using rabbit erythrocytes (RaE). Sera from 20 healthy subjects were studied. Values are given in per cent of the reference.

description of the method has been given (Truedsson & Sturfelt 1983). In short, the assay was performed with low endosmotic agarose, agarose (LE) (FMC Corp., Marine Colloids div., Maine, USA), using 75 mmol/l barbital buffer containing EDTA at 2 mmol/l (VB-EDTA). Polyethylene glycol 6000 was added to the gel at 5% (w/v), and anti-D antiserum at 0.07% (v/v). Electrophoresis was carried out for 5 h at 20 V/cm. After repeated washing in 0.15 mol/l NaCl, the gel was incubated for 30 min with peroxidase labelled anti-rabbit immunoglobulin. After further washing the immunoprecipitates were then stained by adding the substrate of peroxidase - viz., 3-amino-9-ethylcarbazole (Sigma Chemicals, St. Louis, Mo., USA).

Functional assays for D. A HIG assay for D was performed essentially as described by Martin *et al.* (1976); minor modifications were done as earlier described (Truedsson & Sturfelt 1983). The sera tested were

applied diluted 1/2 in veronal-buffered saline with 5 mmol/l Mg^{2+} and 10 mmol/l EGTA (VBS- Mg^{2+} -EGTA).

D activity was also estimated by the tube assay described by Lesavre *et al.* (1979), based on hemolysis of rabbit erythrocytes.

The D activity present in test samples was expressed in per cent of that in the reference serum.

Statistical evaluation. Because of the positively skewed distribution of the values for D concentration, geometric means and 95% geometric reference areas were given.

Correlations and regression lines were calculated by the method of least squares. Logarithmic transformation of data was performed for calculation of age and sex differences. Significance of differences between paired data was calculated using Student's t-test, and reproducibility according to Weeke (1973).

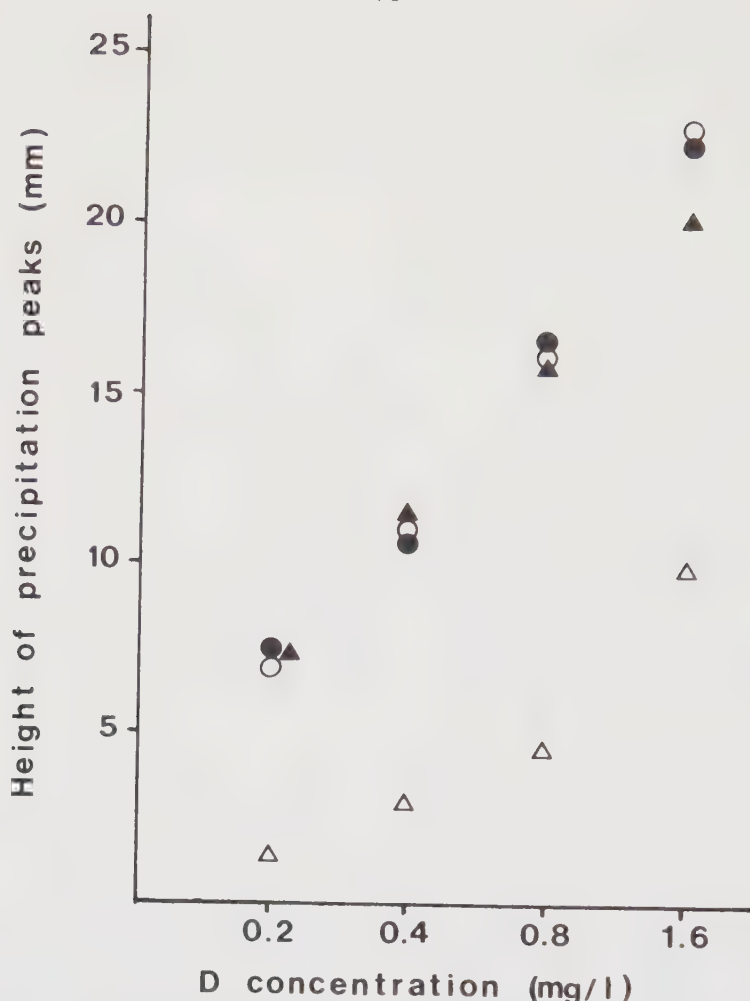


Fig. 3a. Measurement of D protein by EAE. NHS (circles) and purified D (triangles) were diluted in VB-EDTA (open symbols) and RD (filled symbols).

RESULTS

D Concentrations in Normal Sera

D levels were determined in 144 normal sera by the EAE and by the HIG assay for D. The results are given in Table 1. Among the sera studied the geometric mean concentration of D was compared in males and females. In males the mean concentrations of D was 108% by HIG and 105% by EAE and in females 98% in both assays. The difference between the mean D values in the two groups was barely significant ($p < 0.05$) when determinations were made with HIG while the difference between the EAE values was not significant.

To assess influence of age on D serum concentration, the mean D value of 72 normal blood donors aged 19–40 was compared to that of 72 blood donors aged 40–61 years. The geometric mean

values for the two groups were 103 and 105 with EAE and 102 and 109 with HIG, respectively. These differences did not reach significance ($p > 0.1$).

In the sera from 29 healthy children (Table 1), D values were lower than in sera from adults ($p < 0.001$).

Sera from 6 elderly subjects showed D concentrations (range 80–157% with EAE and range 91–174% with HIG), which were within the range for the healthy blood donors.

Comparison between Immunochemical and Functional Quantitation of D

Figure 1 shows the relationship between the values obtained by the two methods. The equations $y = -184.6 + 2.78x$ and $y = 27.91 + 0.77x$, where x denotes the EAE estimates, give the mathematical

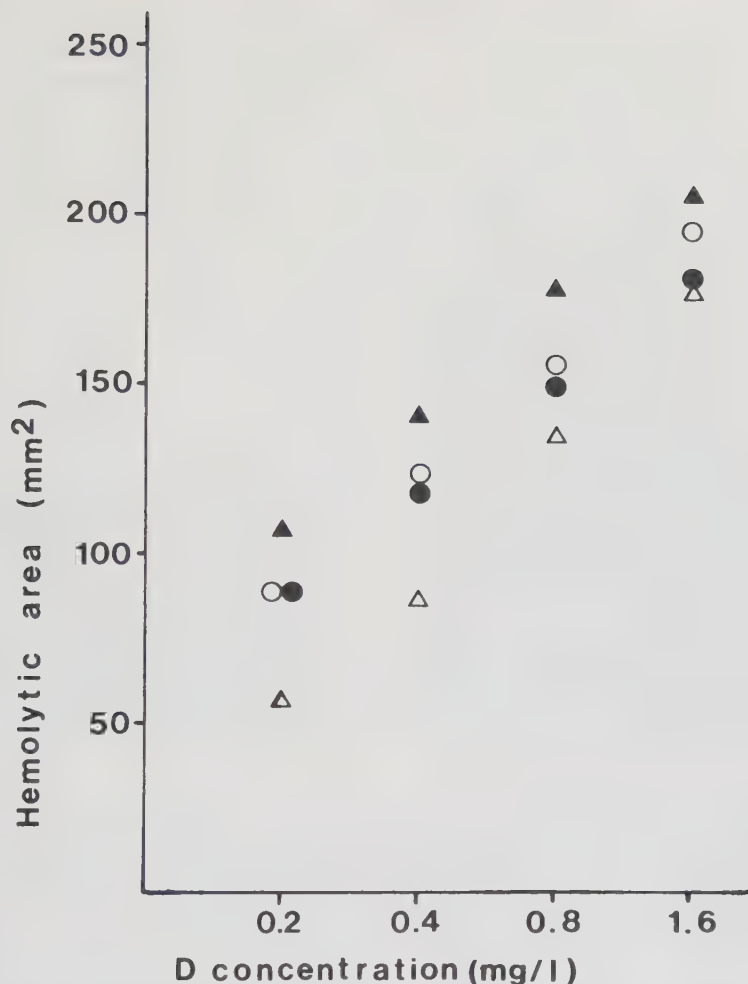


Fig. 3b. Measurement of D hemolytic activity by the HIG assay for D. The same symbols as in figure 3a are used.

relationship between D values in per cent of the reference. Functional and immunochemical values were correlated, $r = 0.52$ ($p < 0.001$). The differences (paired t-test) between the values obtained with the two methods were not statistically significant. In the high range, the functional assay gave higher values than the immunochemical method; and within the low range, functional D values tended to be lower than those determined immunochemically. These differences were not significant, however, nor was the difference between mean D values as estimated with the two methods.

Twenty normal sera were selected from high, low and mid-range areas, as determined by the EAE, for further comparison of functional and immunochemical methods for determining D.

The two hemolytic assays gave similar values with a marked correlation ($r = 0.81$) (Fig. 2). The D

values obtained by EAE showed the same relationship to the D activity found with the two hemolytic assays.

Duplicate determinations of the 20 sera were carried out to assess the reproducibility of the EAE and the HIG assay for D. The variation coefficient for the EAE was 9 % and for the HIG assay 23 %.

Influence of Diluents

Normal serum (NHS) and purified D at normal serum concentration (1.6 mg/l) were serially diluted in VB-EDTA and RD. Simultaneous estimations were performed with the EAE and with the HIG assay (Figs. 3a and 3b). Fairly linear dose-response curves were obtained with both assays.

When the D preparation was diluted in VB-EDTA, low and often sharply pointed immunoprecipitates were found, different from those obtained

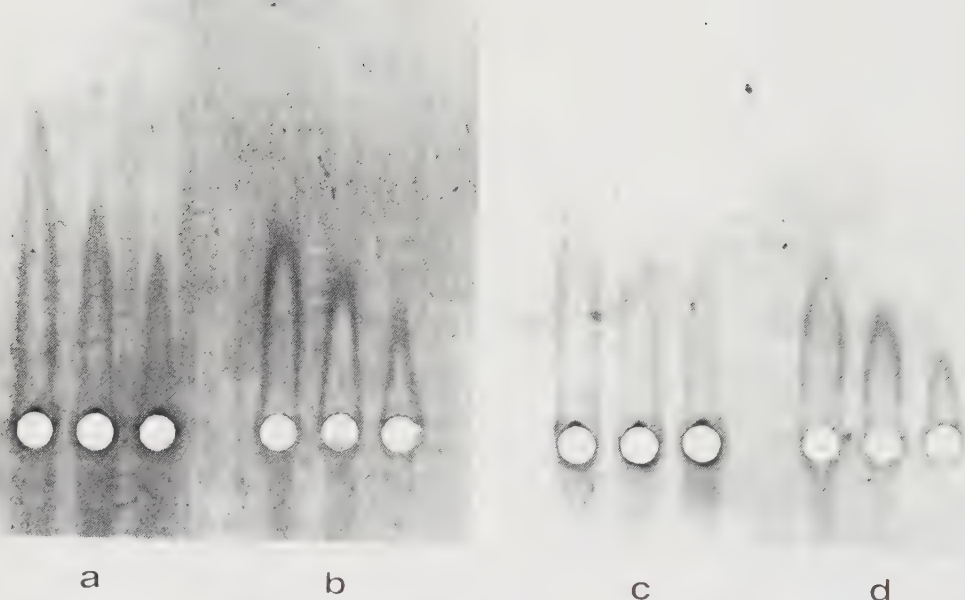


Fig. 4. Immunoprecipitates obtained by EAE with serum diluted in RD (a) and VB-EDTA (b) from a patient with high D level (800% of the reference). For comparison purified D was added to RD at 8-fold the normal serum concentration and then diluted in RD (c) and VB-EDTA (d). Reading from the left, the samples were tested at dilutions 1/8, 1/16 and 1/32.

with normal sera. However, purified D diluted in RD gave immunoprecipitates which were similar to those obtained with normal sera. EAE performed with purified D, in the presence of HSA (40–60 g/l) and with normal sera, gave immunoprecipitates that were almost indistinguishable.

When pathological sera with very high D levels were diluted in buffer the immunoprecipitates obtained were proportionately lower than those found with corresponding dilutions in RD (Fig. 4).

DISCUSSION

In this study D was quantitated in normal sera by an electroimmunoassay with high sensitivity made possible by enzyme amplification. The reproducibility of this immunochemical method was superior to that of the commonly used functional assay for D.

The normal range of D was given by immunochemical and functional measurements and agreed with that in a previous report by *Wilson et al.*

(1979) in which a HIG assay was used. In the 29 sera from healthy children, lower D values were found than for adults, in contrast to the previous finding of increased D serum concentrations in newborns (*Johnson et al.* 1983). The present results indicate, however, that concentrations of D in adult sera are little influenced by age or sex.

In general, good agreement was found between the values obtained by the EAE and by the HIG assay. The differences between values obtained by the two methods were not statistically significant.

Comparison of functional and immunochemical measurements revealed that, in the low range, D values were often higher as estimated by EAE than by two functional assays, and that they were lower in the high range. Similar discrepancies between the results of functional and immunochemical methods reported for C2 may be explained by the presence of nonfunctional C2 protein (*Gibson et al.* 1976) but it is not known whether nonfunctional forms of D protein exist in serum.

Serial dilution of NHS yields linear dose-response curves in both the EAE and the HIG assay. When

purified D was diluted in buffer, values obtained by the EAE were low compared with those found when the D preparation was diluted in RD. In sera with D within the normal range, dilution of sera in buffer is permissible. In pathological sera with high D levels, however, falsely low D values were obtained by EAE on extended dilution of the sera in buffer. Thus it might be advisable to dilute sera to be tested in suitable protein solutions. The present data indicate that RD, or possibly HSA can be used.

It has been established that in the alternative C3 convertase activation, D can be replaced by trypsin (Brade *et al.* 1974) and by kallikrein (DiScipio 1982). The presence of such proteases in pathological sera might result in falsely increased D values when a functional assay is used. Thus immunochemical determination may constitute a more specific means of determining D in clinical samples.

This work has been supported by grants from the Swedish Medical Research Council (B83-16X-68), the Medical Faculty at the University of Lund, Konung Gustaf V's 80-årsfond, Riksförbundet mot Reumatism, Greta och Johan Kocks Stiftelser, Föreningen för Bistånd åt Vanföra i Skåne, and Alfred Österlunds Stiftelse.

REFERENCES

- Brade, V., Nicholson, D., Bitter-Suermann, D. & Hadding, U.: Formation of the C3-cleaving properdin enzyme on zymosan. *J. Immunol.* 113: 1735-1743, 1974.
- DiScipio, R. G.: The activation of the alternative pathway C3 convertase by human plasma kallikrein. *Immunology* 45: 578-595, 1982.
- Fearon, D. T., Austen, F. & Ruddy, S.: Properdin factor D: Characterization of its active site and isolation of the precursor form. *J. Exp. Med.* 139: 355-366, 1974.
- Gibson, D. J., Glass, D., Carpenter, C. B. & Schur, P. H.: Hereditary C2 deficiency: diagnosis and HLA gene complex associations. *J. Immunol.* 116: 1065-1070, 1976.
- Johnson, U., Truedsson, L. & Gustavii, B.: Complement components in 100 newborns and their mothers determined by electroimmunoassay. *Acta path. microbiol. immunol. scand. Sect. C*, 91: 147-150, 1983.
- Lesavre, P. H., Hugli, T. E., Esser, A. F. & Müller-Eberhard, H. J.: The alternative pathway C3/C5 convertase: chemical basis of factor B activation. *J. Immunol.* 123: 529-534, 1979.
- Lesavre, P. H. & Müller-Eberhard, H. J.: Mechanism of action of factor D of the alternative complement pathway. *J. Exp. Med.* 148: 1498-1509, 1978.
- Martin, A., Lachmann, P. J., Halbwachs, L. & Hobart, M. J.: Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. *Immunochemistry* 13: 317-324, 1976.
- Truedsson, L., Sjöholm, A. G. & Laurell, A. B.: Screening for deficiencies in the classical and the alternative pathways of complement by hemolysis in gel. *Acta path. microbiol. scand. Sect. C*, 89: 161-166, 1981.
- Truedsson, L. & Sturfelt, G.: Human factor D of the alternative pathway: purification and the quantitation by enzyme amplified electroimmunoassay. In press. *J. Immunol. Methods*.
- Weeke, B.: Rocket immunoelectrophoresis. *Scand. J. Immunol.* 2: suppl. 1: 37-47, 1973.
- Wilson, W. A., Thomas, E. J. & Sissons, J. G. P.: Complement activation in asymptomatic patients with sickle cell anemia. *Clin. exp. Immunol.* 36: 130-139, 1979.



COMPLEMENT COMPONENTS IN 100 NEWBORNS AND THEIR MOTHERS DETERMINED BY ELECTROIMMUNOASSAY

ULF JOHNSON¹, LENNART TRUEDSSON¹ and BJÖRN GUSTAVII²

¹ Department of Immunology, Institute of Medical Microbiology, University of Lund and ² Department of Obstetrics and Gynaecology, University of Lund, Sweden

Johnson, U., Truedsson, L. & Gustavii, B. Complement components in 100 newborns and their mothers determined by electroimmunoassay. *Acta path. microbiol. immunol. scand. Sect. C, 91*: 147-150, 1983.

Samples of blood were obtained from 100 healthy full-term women in labour and, after delivery, from the umbilical cord of their infants. By electroimmunoassay, complement components were quantitated in serum (C1q, C1r, C1s, C1 IA, C2, P, D, I, H, C6 and C7) or in EDTA-plasma (C4, C3, B, C5, and C8). The concentration of C7 in cord serum was twice that found by others using a functional assay. Concentrations of C1r, I and C6 in the cord sample were 50-60 per cent of those in healthy blood donors used as reference, and that of D was about 130 per cent. The cord serum and plasma concentrations of the remaining components agreed with previously reported values. The maternal levels of C2, C4, C3, B, H, C5 and C6 were 40-60 per cent higher than those of the reference.

Key words: Complement components; cord levels; pregnancy; electroimmunoassay.

U. Johnson, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

Received 29.x.82 Accepted 12.xi.82

Where there is a risk of an inherited disorder within the complement system in the newborn, it may be of importance to analyse single complement components soon after delivery. Such determinations may also be useful in revealing a septicaemia in the newborn infant with uncharacteristic signs of illness.

Other workers have attempted to establish reliable reference values of complement components in the newborn. However, the materials have usually been limited and methods and references have varied from one study to another (cf. Johnston *et al.* 1979). In addition, there seems to be no previous report on the levels of C1r, I, D and C6.

In the present study, we have measured, by electroimmunoassay, the concentration of sixteen complement components (C1q, C1r, C1s, C1 IA, C2, C4, C3, P, B, D, I, H, C5, C6, C7 and C8) in

serum or EDTA-plasma obtained from the umbilical cord of 100 newborn infants. The same components were also quantitated in serum and EDTA-plasma from the mothers.

MATERIAL AND METHODS

Samples of blood were drawn from 100 healthy full-term women in labour. After delivery, but before expulsion of the placenta, samples of blood were collected by free flow from the pendant umbilical cord. One portion of each sample was collected without additive, the other with addition of 5 mmol/l Na₂EDTA. All samples were centrifugated and frozen in aliquots within 8 hours and stored at -80 °C until analysed.

Of the newborn infants, 54 were males and 46 were females.

Reference pools of serum and EDTA-plasma. The results of the quantitation are expressed as percentages of a serum and of an EDTA-plasma reference pool obtained

from 100 apparently healthy blood donors, assuming the pools to contain 100 per cent of the complement component investigated.

Electroimmunoassay. The components C1q, C1r, C1s, C2 and P were measured as described by Laurell *et al.* (1978); C1 IA, C4, C3, B and C5 as described by Sjöholm (1975).

Factor I, H, C6 and C7 were quantitated in serum, the samples being diluted in 75 mmol/l barbitol-HCl buffer, pH 8.6, containing 2 mmol/l Na₂EDTA. The same buffer was used in the electrophoresis, which was run for 6 hours at 20 V/cm using monospecific antisera.

C8 was determined in EDTA-plasma, the samples being diluted in 75 mmol/l barbitol-HCl buffer; pH 8.6, containing Na₂EDTA (2 mmol/l). The electrophoresis was run in the same buffer using a mixed agarose containing 7 parts 0.6% (w/v) Agarose ME (Marine Colloids, Inc., USA) and 3 parts 1% (w/v) Litex agarose, batch AGS 131A (Litex a/s, Denmark) to obtain sharply defined immunoprecipitates. The electrophoresis was run for 20 hours at 8 V/cm using a monospecific rabbit antiserum.

Factor D was estimated by using an enzyme amplified electroimmunoassay. A detailed description of the procedure is given elsewhere (Truedsson & Sturfelt 1983). Briefly, the electroimmunoassay was run as described by Laurell (1972) using 75 mmol/l barbitol-HCl buffer, pH 8.6, containing 2 mmol/l Na₂EDTA. Five per cent (w/v) polyethylene glycol (PEG) 6000 was added to the agarose gel (Agarose LE, Marine Colloids, Inc., USA). A monospecific rabbit antiserum was used and the electrophoresis was run at 20 V/cm for 5 hours. The amplifying technique was principally that described by Löfberg & Grubb (1979). After washing, the gel was incubated with peroxidase-labelled swine antibodies against rabbit IgG (Dakopatts a/s, Denmark). The washing was repeated and the immunoprecipitates were visualized by adding a substrate of peroxidase, viz. 3-amino-9-ethylcarbazole (Sigma Chemical Company, USA) to the gel.

The method error in electroimmunoassay is presented elsewhere (Sjöholm 1975; Laurell *et al.* 1978). The normal ranges for factor D, C6 and C7 are calculated from a normal material comprising 100 blood donors. The adult range given for C8 is that of Wahn *et al.* (1981). The normal adult ranges for the rest of the components investigated are those of Sjöholm (1975) and Laurell *et al.* (1978) and based on quantitation by electroimmunoassay.

Statistics. The arithmetic mean and the standard deviation were calculated for each component. Logarithmic transformation of the values was done to compensate the positively skewed distribution displayed by several components. The geometric 95 per cent confidence area for each component is given. Student's *t*-test for paired observations was used on the transformed values to calculate statistical significance.

RESULTS

The results of the quantitation of the 16 complement components in serum or EDTA-plasma from the 100 newborn infants and their mothers are given in Table 1. The concentrations of certain components (C4, B, D and C7) displayed a positively skewed distribution. The concentrations of the remaining components were almost normally distributed.

In the cord samples, all factors but D and C7 showed mean values below those of healthy adults. The lowest concentrations were those recorded for factors P, C6 and C8 (46, 44 and 36 per cent of the adult level, respectively). Unlike the rest of the components investigated, factor D showed higher concentrations in the cord samples (124%) than in the maternal ones (86%). The C7 values were higher in both cord and maternal samples than in the references.

In the maternal samples, the majority of the components had higher mean levels than the reference material. Exceptions were C1 IA, P and D, that showed lower levels, while C1q and C1r values matched those of the reference.

No statistical significant differences between the mean for each component in male and female newborn infants were observed. The difference between the levels in cord and maternal samples was significant at the $p < 0.001$ level for all components except C7. The components C2, C3, B, I, H, C6 and C8 showed high (more than 60 per cent) discrepancy between cord and maternal samples.

The mean albumin level was 90 per cent of the reference level in the cord samples, and 76 per cent in the maternal samples.

DISCUSSION

The immunochemically determined concentration of factor C7 in cord samples was approximately double that reported by Adolfini & Beck (1975), who used a functional assay for the determination. C7 was the only factor showing raised concentrations in both cord and maternal samples, as compared to the reference. We are not aware of any published report on the cord sample concentrations of the factors C1r, D, I and C6. Of these components factor D had a concentration of about 130 per cent of the controls, whereas the maternal mean value was below that of the reference. The reason for the raised mean value for D in the cord samples is not known. The remaining components showed mean values in cord samples that agreed with those previously reported.

TABLE 1. *The Concentration of Complement Components in Serum or EDTA-plasma from (1) Controls (Healthy Blood Donors), (2) Pregnant Women in Labour, and (3) Newborn Infants (Cord Samples)*

Component	Arith. mean	1 stand. dev.	Geom. mean	Geom. 95 % conf. area
Clq:1	102	13	101	78-131
Clq:2	91	14	90	66-123
Clq:3	70	14	69	47-101
Clr:1	98	17	97	71-133
Clr:2	102	21	99	64-155
Clr:3	60	14	59	38- 91
Cls:1	106	15	105	79-139
Cls:2	121	25	118	78-181
Cls:3	74	14	73	51-105
C $\bar{I}$ IA:1	107	21	105	72-153
C $\bar{I}$ IA:2	86	16	85	58-123
C $\bar{I}$ IA:3	66	12	65	46- 92
C2:1	112	21	111	77-159
C2:2	153	29	150	97-232
C2:3	87	18	86	60-124
C4:1	111	38	105	53-207
C4:2	141	51	132	59-291
C4:3	76	26	71	34-148
C3:1	101	17	99	72-136
C3:2	159	29	156	108-225
C3:3	89	19	87	57-131
P:1	95	24	92	54-157
P:2	85	18	83	54-130
P:3	48	12	46	29- 74
B:1	98	24	95	59-154
B:2	141	32	137	84-224
B:3	53	15	51	29- 88
D:1	104	16	103	75-141
D:2	88	17	86	59-125
D:3	126	25	124	84-183
I:1	98	22	96	60-152
I:2	127	28	124	76-202
I:3	56	19	53	30- 95
H:1	105	22	103	69-154
H:2	157	33	153	98-239
H:3	78	27	75	42-131
C5:1	114	25	111	73-170
C5:2	147	25	145	103-202
C5:3	90	19	88	58-133
C6:1	101	23	99	63-154
C6:2	150	40	144	81-256
C6:3	50	23	44	15-134
C7:1	101	22	99	64-154
C7:2	137	42	131	69-247
C7:3	124	37	119	65-218
C8:1	103	36	96	45-203
C8:2	133	45	126	66-241
C8:3	39	15	36	17- 76

Donaldson (1966) has reported diminished activity of C1 IA in late pregnancy. In a later publication, van Royen *et al.* (1978) ascribed the decreased activity to a cold promoted activation of coagulation factor VII. In the present study, C1 IA was measured immunochemically and the values were compared with a control treated in the same way as the sample.

Opferkuch *et al.* (1978) found no differences in the mean values of complement components between pregnant women and non-pregnant controls. However, it was not stated during which period of the pregnancy the samples were collected. In our study, all samples were drawn from full-term women in labour. With the exception of C1q, C1r, C1 IA, P and D, the components in these women had higher concentrations than the reference. Whether this is due to hormonal influence on the maternal protein synthesis in late pregnancy requires further investigation.

This work was supported from the Swedish Medical Research Council (B83-16X-68-19C), the Medical Faculty of the University of Lund, Alfred Österlunds Stiftelse, Forssmans Fond and Thorsten & Elsa Segerfalks Stiftelse.

REFERENCES

- Adolfini, M & Beck, S. E.: Human complement C7 and C9 in fetal and newborn sera. *Arch. Dis. Child.* 50: 562-564, 1975.
- Donaldson, V.: Serum inhibitor of C1-esterase in health and disease. *J. Lab. & Clin. Med.* 68: 369-382, 1966.
- Johnston, Jr., R. B., Attenburger, K. M., Atkinson, Jr., A. W. & Curry, R. H.: Complement in the newborn infant. *Pediatrics* 64: 781-786, 1979.
- Laurell, A.-B., Mårtensson, U. & Sjöholm, A.: The development of simple tests for C1q, C1r, C1s, C2 and properdin. In: Opferkuch, W., Rother, K. & Schultz, D. R. (Eds.): *Clinical aspects of the complement system*. Georg Thieme Publishers, Stuttgart 1978, pp. 12-14.
- Laurell, C.-B.: Electroimmunoassay. *Scand. J. clin. Lab. Invest.* 29: Suppl. 124, 21-37, 1972.
- Löfberg, H. & Grubb, A.: Quantitation of γ -trace in human biological fluids: indications for production in the central nervous system. *Scand. J. clin. Lab. Invest.* 39: 619-626, 1979.
- Opferkuch, W., Berger, V., Kapp, S., Melchers, G., Prellwitz, W., Ringelmann, R. & Wellek, B.: Standard test values and reproducibility of complement determinations. In: Opferkuch, W., Rother, K. & Schultz, D. R. (Eds.): *Clinical aspects of the complement system*. Georg Thieme Publishers, Stuttgart 1978, pp. 4-11.
- van Royen, E. A., Lohman, S., Voss, M. & Pondman, K. W.: C1 inactivator and cold promoted activation of factor VII. *J. Lab. Clin. Med.* 92: 152-163, 1978.
- Sjöholm, A. G.: Complement components in normal serum and plasma quantitated by electroimmunoassay. *Scand. J. Immunol.* 4: 25-30, 1975.
- Truedsson, L. & Sturfelt, G.: Human factor D of the alternative pathway: purification and the quantitation by enzyme amplified electroimmunoassay. Unpublished results.
- Wahn, W., Rother, U., Rauterberg, E. W., Day, N. K. & Laurell, A.-B.: C3b Inactivator deficiency: association with an alpha-migrating factor H. *J. Clin. Immunol.* 1: 228-233, 1981.



ISBN 91-7222-690-0